NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1994

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director

Period Covered: 20 September, 1993, through 19 September, 1994

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

The Neuroimmunology of Stress, Injury, and Infection

Project Directors:
Bryan Gebhardt, Ph.D.
Daniel Carr, Ph.D.

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Professor of Ophthalmology, Biochemistry and Molecular Biology and Neurology

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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13. ABSTRACT *(Maximum 200 words)*

The LSU Neuroscience Center is a comprehensive, multidisciplinary, and transdepartmental entity that unites fundamental neurobiology and the clinical neurosciences in the common goal of elucidating the workings of the brain and contributing to the treatment of currently incurable diseases of the nervous system. The objective of the present program is to find solutions to neuroscience-related problems of interest to the U.S. Army Medical Research and Development Command. The program is focused on exploiting novel neuroprotective strategies that lead to prevention of and repair after neural injury. Converging approaches using state-of-the-art tools of cell biology, neurochemistry, neuroimmunology, neurophysiology, neuropharmacology, molecular biology and virology are proposed. Over the next four years, this program aims to: 1) carry out seven research projects in the basic and clinical neurosciences; 2) expand central, shared facilities with the addition of highly specialized instrumentation not currently available to our scientists; 3) develop laboratory space to permit the physical consolidation and coordination of this research effort; and 4) institute a coordination unit to monitor, facilitate, and administrate the cooperative research programs, as well as to meet the associated budgetary, human resources, facilities, and communications needs for the attainment of the proposed program goals.

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This Technical Report covers the progress made in the first year of this Cooperative Agreement in one project of the original proposal. We hope that this format of the report will facilitate its handling. The table of contents for all the projects has been included in each volume as well as letters from members of the External Advisory Committee of the LSU Neuroscience Center who have conducted an initial review of the work done supported by this Cooperative Agreement.

Nicolas G. Bazan, M.D., Ph.D.
Director, LSU Neuroscience Center
Program Director, USAMRDC Cooperative Agreement

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Roger Beuerman, Ph.D.

Participating Scientists: Claude A. Burgoyne, M.D.
Emily Varnell
Mandi Conway, M.D.

Manuscripts

1. Chew SJ, Beuerman RW, Kaufman HE (in press) Real-time confocal microscopy of keratocyte activity in wound-healing after cryoablation in rabbit corneas. *Scanning* 16.

"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury" . . . . . .

Project Directors: Prescott Deininger, Ph.D.
Nicolas G. Bazan, M.D., Ph.D.

Participating Scientists: Julia Cook, Ph.D.
Haydee E. P. Bazan, Ph.D.
William C. Gordon, Ph.D.
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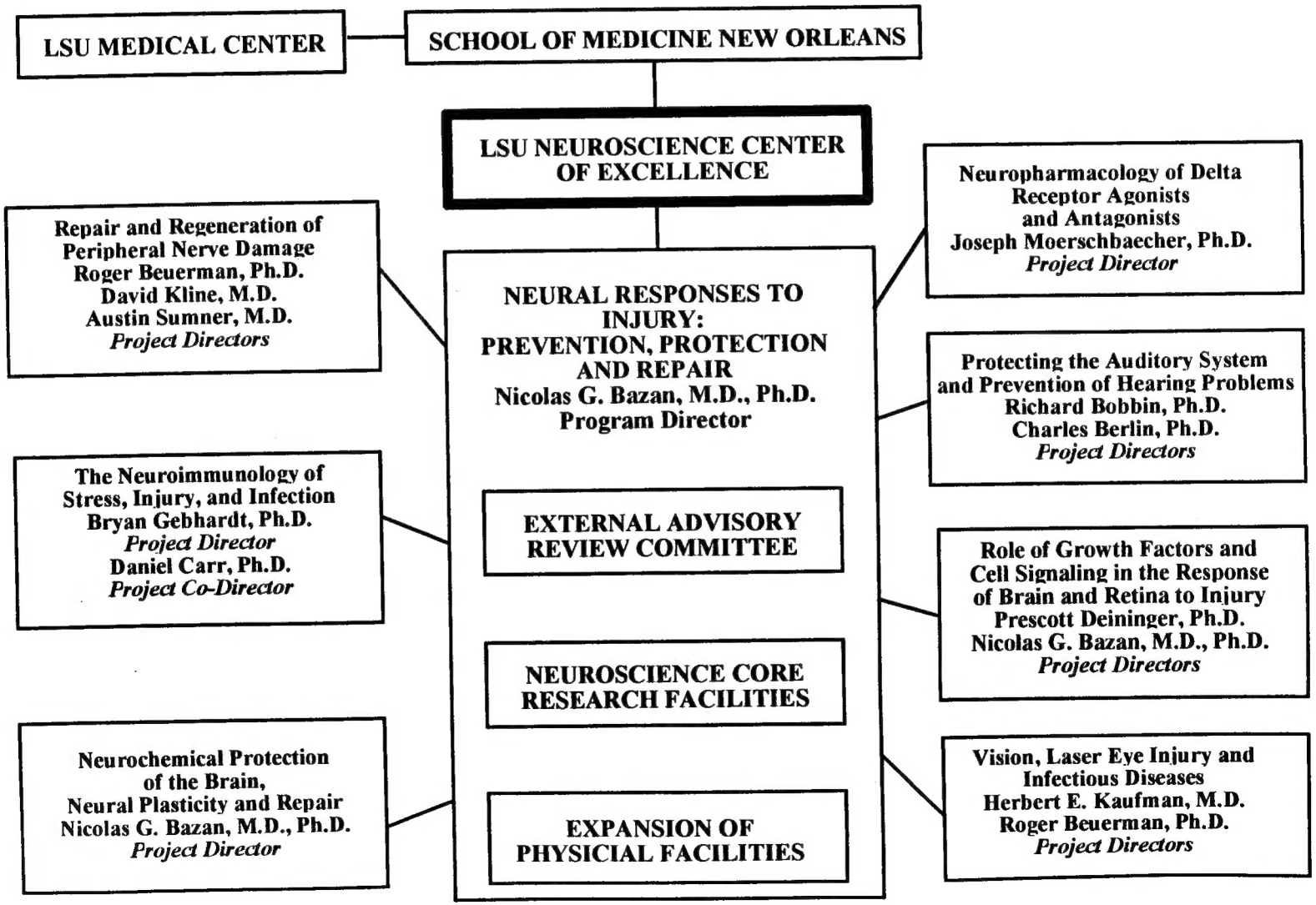
Cooperative Agreement Between the US Army Medical Research and Development Command and The LSU Neuroscience Center of Excellence

DAMD17-93-V-3013 20 September, 1993 - 19 October, 1997 $13,860,000



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SCHOOL OF MEDICINE IN NEW ORLEANS
Louisiana State University
Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234
Telephone: (504) 568-6700
Telefax: (504) 568-5801

Neuroscience Center
Office of the Director

19 October, 1994

Commander
U.S. Army Medical Research and Development Command (USAMRDC)
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21702-5012

Re: Annual report, Cooperative Agreement No. DAMD17-93-V-3013
Neural Responses to Injury: Prevention, Protection, and Repair

Dear Sir,

Please find enclosed the original and five copies of the first annual report for the Cooperative Agreement, referenced above, between the USAMRDC and the Louisiana State University Medical Center School of Medicine, Neuroscience Center of Excellence. This report represents the research carried out during the first year of this agreement (20 September, 1993, to date). It is organized per project, each corresponding to a chapter of the original application.

In addition to the research conducted in the first year of this agreement, the planning for the two additional floors of research space which are to be added to the Lions/LSU Clinics Building, 2020 Gravier Street, New Orleans, LA, has been completed, including all specifications necessary for the start of bidding. Enclosed is one copy each of the program manual (1 vol.) and the project manual (3 vols.) which has been generated by Cimini, Meric and Duplantier, Architects and Planners, for bidding purposes. It should be noted that there will actually be three floors constructed in this one project, two as funded by this Cooperative Agreement and one which is funded by LSU to be used by the School of Medicine for other purposes.

As planned, I arranged to have three meetings between the LSU investigators and their counterparts in the Army to provide program briefings for the work that they were planning to conduct under this agreement as well as to exchange ideas and information of mutual interest. The agendas for each of these meetings are enclosed. These provided both the LSU scientists and those of the Army the opportunity to discuss the work being done, the direction, and the significance to problems of interest to the Department of Defense.

On 2 December, 1993, several of our investigators, excluding the Auditory and Laser/Vision groups, met at the Walter Reed Army Institute of Research, Washington, D.C., with Drs. Frank Tortella, Joseph Long, Mark DeCoster and Jit Dave. These discussions revolved around the neurochemical and neuropharmacological aspects of the program project and provided a forum for the Army scientists tobegin interactions and exchange of information with our investigators.

On 31 January, 1994, the LSU auditory physiology group, represented by Drs. Charles Berlin and Richard Bobbin, and I met at Fort Rucker, AL, with Dr. Kent Kimball and Dr. Ben T. Mozo. These meetings involved presentations and discussions about the protection of the auditory system and prevention of hearing problems in humans.

The LSU investigators involved with the vision research, composed of Dr. Herbert Kaufman, Dr. Roger Beuerman and myself, met on 7 February, 1994, at Brooks Air Force Base, San Antonio, TX. These scientists and those of the Ocular Hazards Research Unit of the US Army Medical Research Detachment made presentations and conducted discussions focused on protection from, repair of, and prevention of laser injuries, specifically to the eye. Each of these information exchanges provided very useful direction and advice for the LSU investigators. These workshops will be conducted annually for the term of this agreement.

At the end of the first year of this program, as planned, I requested that two of the members of the External Advisory Committee of the LSU Neuroscience Center, Dr. Dennis W. Choi, Jones Professor and Head of the Department of Neurology, Washington University School of Medicine, and Dr. Fred Plum, Anne Parrish Titzell Professor and Chairman of the Department of Neurology, Cornell University Medical College, provide a critical review and a written report of the progress of the research accomplished under this Cooperative Agreement. Dr. Choi was given a copy of this annual report and subsequently made a site visit on 15 September, 1994, to the LSU Neuroscience Center. (The agenda for his meeting is attached.) At that time he met with a number of the investigators and administrators involved with whom he discussed many facets of the research being performed under this Agreement. His opinion of the work being done is attached.

Dr. Fred Plum made a site visit on 26 September, 1994, having also been provided previously with a copy of this annual report. He was also given the opportunity to examine the research and other progress made under this agreement and his written critique is also attached. Please note that, near the end of his letter (bottom of page two, first four paragraphs of page 5), Dr. Plum also included a description of projects not directly supported by the Cooperative Agreement but which are very positively impacted by any support of Neuroscience projects. The

reviewers were very complimentary of the positive consequences resulting from this support.

We are very pleased with the progress that has been made. We would like to thank you for the assistance you have given us. Please let me know if there is any further information that I can provide you.

Sincerely,

Nicolas G. Bazan, M.D., Ph.D.
Villere Professor of Ophthalmology,
Biochemistry and Molecular Biology,
and Neurology
Director, LSU Neuroscience Center

NGB/eht
enclosures

JOINT WORKSHOP ON "NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION AND REPAIR"

Sponsored by the LSU Neuroscience Center and Walter Reed Army Institute of Research, Department of Medical Neurosciences

December 2, 1993
Building 40, Room 2133

"Overview of LSU Program" **N. Bazan**	9:00
"Repair and Regeneration of Peripheral Nerve Damage" **R. Beuerman, D. Kline, J. England**	9:20
"The Neuroimmunology of Stress, Injury and Infection" **D. Carr**	10:10
Break	10:20
"Neurochemical Protection of the Brain, Neural Plasticity and Repair" **N. Bazan**	10:40
"Neuropharmacology of Delta Receptor Agonists and Antagonists" **J. Moerschbaecher**	11:15
"Stress and the Dopamine System" **J. Rao**	11:45
Box Lunch Served ($2.00 each)	12:00
"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury **N. Bazan and J. Cook**	12:10
"An Overview of Neuropharmacology Research at WRAIR on Nervous System Injury and Protection" **Frank Tortella**	13:00
"Animal Models of Spinal Cord Injury and Mechanisms of Blood Flow Changes" **Joseph Long**	13:30
"Evaluation of Excitatory Amino Acids in Neuronhal Cell Culture" **CPT DeCoster**	13:50
"Molecular Biology of Nervous System" **Jit Dave**	14:10
Overall Discussion	14:30
Adjourn	15:00

zwp51\army\workshop.al

Joint Workshop on Neural Responses to Injury:
Prevention, Protection and Repair
Walter Reed Army Institute of Research, Dept. of Medical Neuroscience
U.S. Army Aeromedical Research Laboratory, Fort Rucker, AL
SCHEDULE FOR JANUARY 31, 1994

January 30
12:00 PM - depart New Orleans by car
Hotel: **Comfort Inn, 615 Boll Weevil Circle, Enterprise, AL 36330**
Tel. 205-393-2304, Fax. 205-347-5954

January 31
Visiting - Dr. Kent Kimball, Director, Plans and Programs, USAARL
Dr. Ben T. Mozo, Research Physicist, USAARL
Fort Rucker, AL 36362-5292
Tel. (205) 255-6917, Fax. (205) 255-6937

9:00 AM - Welcome
9:20 AM - Overview of LSU Program - **Nicolas G. Bazan**
9:45 AM - Protection the Auditory System and Prevention of Hearing Problem via Efferent Activation in Humans - **Charles Berlin**
10:30 AM - Break
11:00 AM - Prevention of Hearing Problems in Animals - **Richard Bobbin**
12:00 PM - General Discussion and Lunch
13:00 PM - Adjourn

OCULAR HAZARDS RESEARCH
U.S. ARMY MEDICAL RESEARCH DETACHMENT
7914 A DRIVE (Bldg 176)
BROOKS AIR FORCE BASE, TEXAS 78235-5138

February 7, 1994

Leave New Orleans on Continental flight #1445 at 6:00 PM, arrive San Antonio on Continental flight #1120 at 8:53 PM.

Hyatt Regency San Antonio
123 Losoya St., San Antonio, TX 78205
Confirmation #HY0000605552

February 8, 1994

8:30 *Overview of USAMRD program*
Bruce Stuck, Director, USAMRD

8:45 *Review of Accidental Laser Exposures and Human Tissue Response*
Donald Gagliano, Commander, USAMRD

9:00 *Overview of LSU Program*
Nicolas G. Bazan, Director, LSU Neuroscience Center

9:10 *The Program: Vision, Laser Eye Injury, and Infectious Diseases*
Herbert Kaufman, Chairman, Ophthalmology Dept. LSU

10:00 *Confocal Approach to Cellular Reactions in Wound Healing and of the Lamina Cibrosa.*
Roger Beuerman of the LSU Neuroscience Center

10:30 **BREAK AND LAB TOUR**

10:50 *Neurochemical Protection of the Brain, Neural Plasticity, and Repair*
Nicolas Bazan, Director, LSU Neuroscience Center

11:40 *Basic Fibroblast Growth Factor (bFGF) Treatment of Laser-Injured Retina*
Steven T. Schuschereba, Chief, Biology Section, USAMRD

12:10 *Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury: Focus on the Retina*
Nicolas Bazan, Director, LSU Neuroscience Center

12:50 **LUNCH**

2:50 Depart San Antonio on Southwest flight #803

5:55 Arrive New Orleans on Southwest flight #1055

LETTERS FROM MEMBERS OF THE EXTERNAL ADVISORY COMMITTEE

WASHINGTON UNIVERSITY SCHOOL OF MEDICINE
AT WASHINGTON UNIVERSITY MEDICAL CENTER

NEUROLOGY

Dennis W. Choi, M.D., Ph.D.

Andrew B. and Gretchen P. Jones Professor and Head
Neurologist-in-Chief, Barnes Hospital

October 17, 1994

Nicholas G. Bazan, MD, PhD
Director, LSU Neuroscience Center
School of Medicine in New Orleans
Louisiana State University Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234

Dear Nick:

Thank you for the invitation to visit LSU on September 15 and review early progress made under the LSU Neuroscience Center of Excellence Cooperative Agreement with the U.S. Army Medical Research and Development Command.

You have assembled an impressive array of faculty researchers to study diverse aspects of nervous system injury. Overall, I find the individual projects to be thoughtful and well chosen. With you as director, I am sure that they will be most ably integrated. Your project 3 "Neurochemical Protection of the Brain, Neuroplasticity and Repair" is in my view the clear focal point of the overall program. The identification of new PAF antagonist drugs capable of regulating excitatory synaptic transmission and excitotoxic central nervous system injury, is an attractive and attainable goal. The novel pharmacology theme is also well developed in Dr. Moerschbaecher's Section 4 "Neuropharmacology of Delta Receptor Agonist and Antagonist". Involvement of clinician-investigators in clinical departments, such as Dr. Sumner in Project 1 or Dr. Kaufman in Project 5 are strengths of the program that will enhance its ability to identify human therapeutic interventions.

Progress in the first months of operation appears to be on target. Substantial synergy can be expected between the research programs specifically outlined in this collaborative agreement, and the larger intellectual framework formed the LSU Neuroscience Center of Excellence. Your role as director of both efforts is a vital feature that will ensure maximization of this synergy. In summary, I am most enthusiastic about this LSU-U.S. Army Cooperative Agreement, both for its specific merit and as a prototype mechanism for facilitating effective collaboration between academic and military institutions.

Best regards.

Sincerely,

Dennis Choi

Box 8111
660 South Euclid Avenue
St. Louis, Missouri 63110
(314) 362-7175 • FAX (314) 362-2826

525 EAST 68th STREET NEW YORK N Y 10021

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THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

FRED PLUM, M D , CHAIRMAN
ANNE PARRISH TITZELL PROFESSOR OF NEUROLOGY
CORNELL UNIVERSITY MEDICAL COLLEGE
NEUROLOGIST-IN-CHIEF
THE NEW YORK HOSPITAL- CORNELL MEDICAL CENTER
(212) 746-6141
FAX (212) 746-8532

September 28, 1994

Nicholas G. Bazan, M.D., Ph.D.
LSU Neuroscience Center
2020 Gravier Street
Suite B
New Orleans, LA 70112-2234

Dear Dr. Bazan:

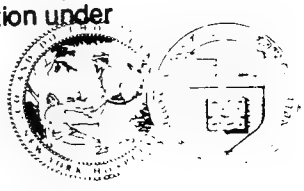
I am pleased to submit this reviewer's report of a Cooperative Agreement between the LSU Neuroscience Center and the US Department of the Army entitled, "Neural Response to Injury: Prevention, Protection and Repair" (henceforth designated as "Injury Study"). The agreement will span four years of effort by the LSU Center; this report describes progress obtained during its first year, extending from September 1, 1993 to August 31, 1994.

Nicholas G. Bazan, M.D., Ph.D. both directs the LSU Neuroscience Center of Excellence and serves as the Program Director of the Injury Study. In addition to Dr. Bazan's personal investigative efforts, seven additional study groups are engaged in research directly related to the Injury Study, as indicated in the administrative diagram attached to this report.

Dr. Bazan's outstanding personal and scientific qualities are the two most important factors in assuring the future success of the LSU-U.S. Army Cooperative Agreement. His leadership and intellectual "taste", as well as his joy in and dedication to brain science penetrate every aspect of the LSU Neuroscience Institute. His enthusiasm has spread to infect his colleagues and many other departments of the Medical School with his high scientific standards and integrity. His knowledge suffuses every dimension of basic neuroscience. His diplomacy and gentle handling of his staff creates their huge loyalty. His energy is contagious. Furthermore, he has the wonderful quality of scientific generosity: always ready to help and encourage others, he is entirely responsible for the continuously improving quality of young persons who are coming to LSU to learn and do important neuroscience.

In addition to the above, Dr. Bazan's specific research is internationally recognized as being of the highest caliber. His personal research contributions to the Injury Study during the past year reflects these high qualities in several ways. They have been published in the most competitively prestigious biomedical research journals. They also add new understandings to both the normal and potentially abnormal effects of the platelet-activating factor (PAF). PAF already is known to be a potent mediator of inflammatory and immune responses. What Bazan and his team now have found is that in low concentrations, PAF transmission may enhance memory and repair mechanisms in brain. Alternately, if released in excessively large concentrations or in combination with certain other molecules, PAF appears capable of causing immune-related tissue damage such as occurs with intense inflammation and/or the induction of genetic prostaglandin synthesis, a step that also may injure brain tissue. This fundamental research emphasizes the complexity and often bidirectional responses that may occur when injury strikes the brain. The results are important and illustrate the difficulties which must be overcome in establishing prevention, protection and repair of brain injuries.

Drs. Bazan and Prescott Deininger have succeeded in developing a series of transgenic mice expressing a dominant mutant of platelet derived growth factor (PDGF). Remarkably enough, the animals thus far have shown no major behavioral alteration under



normal developmental conditions. Their reaction to ischemia, seizures and other circumstances has not yet been tested.

Let me turn now to some of the other, supporting projects: **Drs. R. Benerman, D. Kline and A. Sumner** have made good progress in their studies of neurotrophic factors and other mechanisms in human and experimental neuromas resulting from blunt and crush nerve injuries. Basic fibroblast growth factor (bFGF) was the most prominent factor found in human post-nerve injury neuromas with other specific factors either absent or reaching only very low levels of concentration. More precisely analytic experiments await the analyses of fresh neuronal material from the experimental preparations.

Drs. Herbert Kaufman and Roger Benerman have made brilliant advances using confocal microscopy to examine the cellular details of the human retina. To a degree never before possible they have safely demonstrated in awake human subjects the acute pathophysiology of laser injuries to cornea and their early transformation into fibroblasts. Detailed identification of anterior chamber cells has been possible and current efforts are underway to examine at great magnification the optic disc itself. Ocular fungus and herpes infections can be identified immediately and without introducing foreign substances against the cornea or into the eye. Application of the tool should have an important place in clinically applied military medicine.

During the past year, the investigators also have pursued their earlier discovery that ambient chilling of monkeys latently infected with H. Simplex induces an acute recurrence of cutaneous herpes. Furthermore, chronic ingestion of the beta blocker, propanelol, has been found to ameliorate or prevent the active recurrence. Clinical trials of this important discovery must be pursued as it has important practical aspects.

During the year, the necessary work to establish and equip the glaucoma research laboratory was undertaken. Next year's report can be expected to provide research results from that laboratory.

Dr. Joseph Moerschbaecher and his colleagues in pharmacology have initiated preliminary studies on the influence of delta opoid agonists-antagonists on learning and antinociception. Somewhat surprisingly, the agent damps the CO_2 response of breathing but has no antinociceptive effect. The same investigator is analyzing how anxiogenic drugs affect dopamine neurons in the ventral tegmental area of the rodent brain.

In another preliminary approach, **Drs. H.W. Thompson et al** have initiated experiments passing retroviral gene carriers into the eye with externally applied eye drops, thereby developing a new approach to deliver protection against certain ophthalmologic infections or enhancing the potential success of corneal transplant.

Drs. Richard Bobbin and Charles Berlin, thanks to the DOD grant, have added an excellent postdoctoral student as well as important new equipment to their laboratory. The laboratory's principal subject of interest is to find mechanisms for preventing the audiologic damage produced by intense sound. In guinea pigs, this has been achieved by stimulating calcium-dependent mechanisms in cochlear neurons. In another study, the laboratory has found in human studies that during the delivery of loud, binaural sounds, men and women suppress the noise in opposite sided ears from each other.

The above individual achievements provide only a part of the considerable effort, enthusiasm and success that the U.S. Army grant has brought to the LSU Neuroscience Center of Excellence (NCE). The following steps forward can also be emphasized:

1) Morale in the LSU-NCE rides at high pitch, encouraging scientific collaboration and the generation of new ideas.

2) Funds have been granted to subsidize the necessary equipment and technical personnel to establish a brain bank. Presently, approximately 50 specimens are available in storage with the Center holding good clinical records of the preterminal illness.

3) A program of "starter" grants designed to assist young investigators in conducting merit-deserving, self designed research projects has been initiated.

4) A highly popular state-wide Graduate School outreach summer program has been successfully concluded, attracting a strong interest in neuroscience among gifted college students.

5) An interdisciplinary graduate program in neuroscience was initiated and strongly encouraged by the faculty during 1993-94. As a result, nearly all of the graduate students (including the new entering class) are of very good quality. Indeed, other participating departments say that the Neuroscience graduate students are the best among the LSU biological sciences programs.

Summary. Under the generous auspices of a U.S. Army Cooperative Agreement, the LSU Neuroscience Center of Excellence is not only thriving but headed for far greater future productivity than at any time in the past. The admirable success of the program depends heavily on the foresight, intelligence, creativity and energy of two outstanding scientists, Herbert Kaufman and, especially, Nicholas G. Bazan. Their achievements and those of their colleagues totally warrant continuation of support. Indeed, every indication is that their extramural, non-Army support will continue to grow, making the program stronger and stronger as the years elapse.

One serious problem remains - that of sufficient space in which to do the studies that Dr. Bazan and his colleagues already have conceived so well. Prompt attention to and effective application of must be given to the DOD funds already awarded to construct new research space which will greatly increase the LSU Neuroscience team's opportunities for creative discovery.

I and my colleagues on the External Advisory Board of the LSU Neuroscience Center of Excellence strongly endorse the quality and number of achievements that have come from the U.S. Army-LSU-NCE collaboration. Thanks to strong leadership for the Center and a high degree of internally high morale and interdependence within the Center, it can be anticipated that the Cooperative Agreement will have a major impact on national neuroscience research as well as the specific medical needs of the U.S. Army.

Sincerely,

Fred Plum

Fred Plum, M.D.

FP/moc

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FOREWARD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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(X) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources Commission on Life Science, National Research Council (NIH Publication no. 86-23, Revised 1985).

NA* ( ) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

(X) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Bryan M. Kelhardt

Investigator Signature

*Not Applicable

ANIMAL USE
20 SEPTEMBER, 1993, THROUGH JULY, 1994

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neuroimmunology of Stress, Injury and Infection**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC #
Mouse	270	270	1019

Bryan M. Hebbandt
Investigator Signature

ABSTRACT

The hypothesis on which this investigation is based is that stressors such as transient temperature changes and restraint signal the central nervous system eliciting the release of catecholamines and adrenal steroids which, in turn, affect the immune system resulting in the reactivation of latent viruses. Employing a mouse model of stress-induced reactivation of herpes simplex virus type 1 (HSV-1), we are determining the time course of viral reactivation relative to the alteration of immune parameters including lymphocyte functions and numbers. Specifically, we are correlating the expression of various immunomodulatory cytokine genes with the levels of neuroendocrine monoamines, as well as the activation of the hypothalamic-pituitary-adrenal (HPA) axis and relating these to the reactivation of infectious virus in the nervous system. Alterations in serum corticosterone and shifts in monoamines in the brains, trigeminal ganglia, and brain stems of latently infected and reactivated mice following the application of stress are being studied. Differences between control (not stressed) and stressed animals are being determined relative to the incidence of viral reactivation and the affect of stress on immunological regulation of the reactivation process. The knowledge gained from this investigation will provide an understanding of the interaction between the nervous system, the neuroendocrine system, and the immune system during times of stress at the molecular and cellular levels.

INTRODUCTION

Psychological (e.g., depression) and physical (e.g., restraint) stresses have been shown to suppress normal immune responses (for reviews see Kemeny, 1991; Kiecolt-Glaser and Glaser, 1991). The suppression of immune function following cognitive stress is believed to be mediated predominantly through the activation of the HPA axis resulting in the secretion of adrenal steroids which are selectively immunosuppressive (Cupps and Fauci, 1982; Munck and Guyre, 1991). Stress is also known to activate the sympathetic nervous system which innervates lymphoid organs (e.g., spleen). Cholinergic, peptidergic, and noradrenergic neuronal processes are all found in various lymphoid tissues (for review see Felten and Felten, 1991). Adrenal steroids and catecholamines have been proposed as the primary mediators of altered immunological function following stress. It has been shown that lymphocytes express glucocorticoid (Munck and Leung, 1977) and adrenergic (for review see Madden and Livnat, 1991) receptors. There is also a plethora of data detailing the immunoregulatory affects of these hormones and neuromediators *in vitro* and *in vivo* (Munck and Guyre, 1991; Madden and Livnat, 1991).

With this knowledge regarding the possible nervous system impact on immunological competence, we have proposed to study the neuroendocrine immunological interactions which take place subsequent to episodes of stress and which lead to reactivation of latent herpes simplex virus type 1 (HSV-1). There is a dearth

of information available regarding the stress-induced neurochemical alterations which affect immunological homeostasis.

Dobbs, et al (1993) recently noted that both corticosterone and catecholamines participate in stress-induced suppression of antiviral cellular immunity during primary infection. Our studies are designed to investigate neuroendocrine immunologic interactions during viral reactivation. The results of this study have a direct bearing on stressed personnel who are rendered ineffective due to reactivated latent infection. The experiments described will explore the neuroimmunological events which occur during reactivation of latent HSV-1 infection. The pathogenesis of viral reactivation following the application of physical stress (restraint and thermal) in mice will be investigated. This model has been useful in delineating some of the molecular immunological events which occur during reactivation of the virus in the trigeminal ganglion (Gebhardt, 1993; Sawtell and Thompson, 1992). Other investigators have noted that HSV-1 reactivation may be elicited by psychological or physical stressors (Kemeny, 1991). This study will permit us to better understand the cellular and humoral immune functions which control the pathogenic manifestations of viral infection relative to neuroendocrine activation following stress.

BODY

During the first year of the study we have made significant progress and major new findings regarding the neuroendocrine-immunological regulation of herpes simplex virus type 1 (HSV-1) reactivation from latency. Under the auspices of support provided by USAMRDC Log No. 93148001, we have been able to attract two outstanding graduate students to work on this project and are currently sponsoring at least two undergraduate students who are seriously considering careers in the neurosciences.

Our scientific goals and objectives for the first year were divided into two broad specific aims. The following narrative describes the experimental approaches and results obtained in investigating these specific aims. It will become readily apparent that significant progress has been made including the submission of numerous abstracts, the presentation of data at local and national meetings, and the preparation and submission of manuscripts relating to these observations. The following narrative is divided according to the specific aims for year one of the project.

Specific Aim 1: To determine the effects of a brief period of thermal stress (10 minutes at 43°C) and restraint stress (60 minutes) as indirect mediators of HSV-1 reactivation from neural tissues.

These experiments were designed to allow us to determine the frequency of viral reactivation following moderately stressful events and to establish the baseline from which we can launch a

full-scale assault on the analysis of the neuroendocrine-immunologic interactions which take place during stress-induced viral reactivation. The essential experimental protocol is as described in specific aim 1. Groups of 10 mice in each group, which had been infected with the McKrae strain of HSV-1 by the ocular route 35 days previously were subjected to one of the stress protocols. At 24 hours after the application of the stressor (43°C for 10 minutes or 60 minutes of restraint stress) the groups of mice were sacrificed, the ocular surface swabbed, the globes removed, and the trigeminal ganglia dissected free. Control groups of animals included uninfected, stressed animals, infected animals that were not stressed, and animals which were neither infected nor stressed.

Table 1 below indicates the results of the assays for infectious virus from the experimental and control groups of animals. For the convenience of the presentation of the data, the results obtained with animals subjected to the two different stress paradigms are separated into Tables 1 and 2. It can be seen that the heat stress paradigm induced a higher percentage of reactivations (80%) as compared to the restraint stress model (40%). This experiment was repeated three times in order to confirm the results and to permit detailed statistical analyses. By analysis of variance (ANOVA) the difference between the reactivation of infectious virus in the tears, ocular tissue, and trigeminal ganglia of infected, stressed animals was significantly different from any of the control groups

($P < 0.005$). Thus, the stressors chosen for use in these investigations are bonafide methods of inducing viral reactivation.

TABLE 1: Viral Reactivation Following Heat Stress

Treatment Groups	Presence of Infectious Virus In:		
	Tear Film	Corneas	Trigeminal Ganglia
Infected, stressed	4/10	6/10	8/10
Infected, not stressed	0/10	0/10	0/10
Not infected, stressed	0/10	0/10	0/10
Not infected, not stressed	0/10	0/10	0/10

TABLE 2: Viral Reactivation Following Restraint Stress

Treatment Groups	Presence of Infectious Virus In:		
	Tear Film	Corneas	Trigeminal Ganglia
Infected, stressed	1/10	3/10	4/10
Infected, not stressed	0/10	0/10	0/10
Not infected, stressed	0/10	0/10	0/10
Not infected, not stressed	0/10	0/10	0/10

Specific Aim 2: To determine the neuroendocrine mechanism of stress-induced viral reactivation by measuring corticosterone and monoamine levels in the serum and nervous tissues of latently infected, stressed animals.

The overall goal of the experiments conducted as part of

this specific aim are to determine the types of neuromediators which are involved in viral reactivation and also which are involved in modulating the immune response so as to permit viral reactivation. To determine the possible interactions between latent HSV-1 infection and the stress response of mice, the induction of endocrine and paracrine mediators was compared in four treatment groups: infected, stressed mice; infected, not stressed mice; uninfected, stressed mice; and uninfected, not stressed mice. Using the heat stress paradigm described above, it was found that 24 hours after application of the stressor that serum corticosterone in the infected mice were significantly higher as compared to uninfected mice ($P < 0.05$). The data from a typical experiment in this series is shown in Table 3 below.

TABLE 3: Effect of Heat Stress-Induced Viral Reactivation on Corticosterone Levels*

	HSV-1 Infected	Not Infected	Averages
Stressed	102.3	38.5	70.4
Not Stressed	63.1	52.2	57.7
Averages	82.7	45.3	

Two-way ANOVA:

H_0:

	Probability:
X_{av} (Stressed) = X_{av} (Not Stressed)	$p = 0.28$
X_{av} (Infected) = X_{av} (Not Infected)	$p = 0.015$

*nanograms/ml

It can be seen that there was a statistically significant elevation in corticosterone levels in the infected mice. Additional studies of the monoamine levels in the brain stems of

the experimental and control groups of animals have been conducted 24 hrs after application of the stressor. Catecholamine levels were not found to be significantly altered in any of the treatment and control groups investigated to date (Table 4).

TABLE 4: Effect of Heat Stress-Induced Viral Reactivation on Brainstem Catecholamine Levels*

Treatment Groups	NE‡	SHIAA	DA	5HT
Infected, stressed	931	228	31.3	220
Infected, not stressed	1030	244	88.3	280
Not infected, stressed	742	131	23.2	186
Not infected, not stressed	966	227	56.6	251
One-way ANOVA: $X_a=X_0=X_0=X_a$	p = 0.77	p = 0.58	p = 0.17	p = 0.69

‡NE = norephinephrine; 5HIAA = 5-hydroxyindoacetic; DA = dopamine; 5HT = 5-hydroxytrytophane

*picograms/milligram

Further studies of the catecholamine and corticosterone levels at earlier timepoints and the significance of this response to viral reactivation are in progress.

Our initial investigation into the modulation of the immune response mediated by neuroendocrine mediators as a response to heat stress has produced some meaningful results. Among other things, we have investigated the production of interleukin 6 (IL-

6) at the cellular and molecular level following application of stress to latently infected mice. We have noted that serum concentrations of corticosterone and IL-6 in uninfected, stressed mice were positively correlated (r = 0.87), but were negatively correlated (r = -0.28) in infected, stressed mice. These data are shown in Table 5 below. This observation suggests that an underlying difference exists between the regulation of IL-6 and corticosterone when one compares latently infected and uninfected animals following exposure to the stressor.

TABLE 5: Correlation Coefficients of Serum Corticosterone and IL-6 Levels in Heat Stressed, Infected Animals

Treatment Groups	r Values
Infected, stressed	r = -0.28
Infected, not stressed	r = -0.57
Not infected, stressed	r = 0.87
Not infected, not stressed	r = 0.27
T-test: H_0: $r_{(infected/stressed)} = r_{(not\ infected/stressed)}$	

Molecular biological analysis of the expression of the IL-6 gene in the trigeminal ganglion of stressed latently infected mice compared to stressed, uninfected mice, as well as mice that were not stressed but latently infected, reveal that there is an apparent reduction in the expression of the IL-6 message in the latently infected, stressed animals (Fig. 1). Since, the

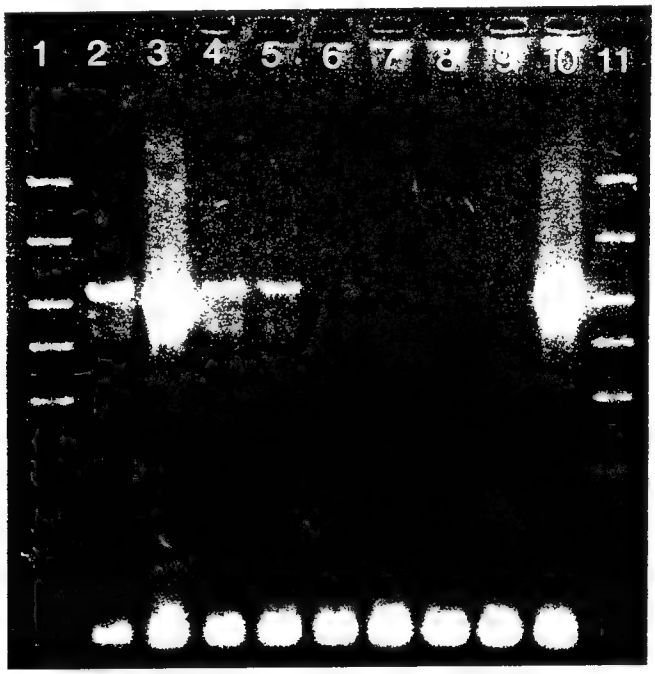

Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) for IL-6 mRNA in trigeminal ganglia. Lane 1 = Molecular weight markers; Lane 2 = Not infected, not stressed; Lane 3 = Not infected, stressed; Lanes 4,5 = Infected, not stressed; Lanes 6-9 = Infected, stressed; Lane 10 = Positive control; Lane 11 = Molecular weight markers.

trigeminal ganglion is one of the sites of latency of HSV-1, we speculate that perhaps the virus can in some way alter the host's production of immunomodulatory mediators including the

pro-inflammatory cytokine IL-6 and, by extension, corticosterone. Currently, competitive PCR is being carried out to quantitate differences between the levels of IL-6 expression in the trigeminal ganglion of the various treatment groups. We can now detect as few as 100 copies of template. Further studies of the role of cytokines (TNF-α, IL-1, and IL-6) and neuroendocrine mediators in viral reactivation during the host immune response are in progress.

Specific Aim 3: To investigate the role of the neuroendocrine system following morphine exposure.

As a direct result of the Department of Defense support for our study, we have been able to provide new insights into the role of morphine-mediated suppression of cellular immunity in mice. These studies have culminated in the submission of four abstracts (Appendix items 1-4), the presentation or planned presentation of our findings at three national meetings, and the submission of four manuscripts (Appendix items 5-8). In addition, pertinent studies are near completion in the cloning of an orphan opioid receptor from lymphocytes which may lead to insights as to the role of opioids (exogenous and endogenous) on the regulation of immunocompetence either constitutively or following bacterial, parasitic, or viral infection. A summary of the findings to date are presented below.

Opioids, such as morphine, are a known chemical stressor that has a detrimental effect on the immune system. In fact, early research by Bryant, Bernton, and Holaday (1987; 1991) of

the Division of Neuropsychiatry of Walter Reed Army Institute showed that morphine was a potent immunosuppressive drug which when administered *in vivo* suppressed mitogen-induced lymphocyte proliferation and delayed-type hypersensitivity reactions through the activation of the HPA axis. To further these initial observations, the P.I. (Gebhardt) and co-P.I. (Carr) began a series of studies to investigate the potential role of the sympathetic nervous system and HPA axis involvement in morphine-induced suppression of cell-mediated immunity concentration on natural killer (NK) activity and the generation of cytotoxic T lymphocytes (CTLs). Both of these effector populations are involved in viral and tumor surveillance making them key figures in reducing the potential incapacitation as a result of viral reactivation or infection. The results of these studies showed that morphine suppressed NK activity through central activation of α-adrenoreceptors (Carr et. al, 1994). Additional studies were conducted on chronic morphine exposure and CTL activation using an allogeneic mouse model. The results of these studies show that chronic morphine exposure significantly suppresses CTL activity following alloimmunization in mice. The suppression is, in part, due to a reduction in intracellular signalling following effector - target cell conjugation as well as the release and synthesis of enzymes associated with the "lethal hit" (Appendix item 5). Using a pharmacological approach, the suppression can be antagonized using the μ-opioid receptor antagonist, β-funaltrexamine (Appendix item 6), but not the δ-opioid receptor

antagonist, BNTX (Appendix item 7). Likewise, serum measurements of corticosterone and DHEA from the vehicle- and morphine-treated mice suggested aberrant adrenal function in the morphine-treated animals (Appendix item 5). The biological significance of these results is substantiated by the study showing that mice infected with HSV-1 and exposed to morphine succumb sooner and with increased frequency, as compared to vehicle-treated, infected mice. These results have helped establish potential mechanisms at a systemic, cellular, and molecular level that may be altered following chronic morphine exposure (Appendix item 8).

The observations showing that morphine-induced suppression of immunocompetence is mediated through central (brain) pathways has led us to investigate the relevance of the hypothesized presence of the opioid receptor(s) on cells of the immune system. Recently, the P.I. and co-P.I. generated oligonucleotide primers specific for transmembrane 3 and 5 and the δ-opioid receptor and used these oligonucleotides to detect potential transcripts in stimulated and unstimulated lymphocytes. By RT-PCR, a product of 381 pb was generated. Subsequent primers were made and used to obtain a full-length cDNA which has >90% sequence homology to an orphan opioid receptor cloned from mouse brain. Northern gel analysis of RNA obtained from splenic lymphocytes indicates the transcripts are present but in reduced numbers relative to brain extracts (Halford, Gebhardt, & Carr, manuscript in preparation). These results will be instrumental in establishing a direct link between the endogenous lymphocyte/macrophage-derived

proopiomelanocortin hormones, the endorphins, and the potential autocrine- or paracrine-oriented feedback on cells of the immune system as well as the potential direct interaction between exogenous opioids (e.g., morphine) and cells of the immune system.

Currently, a kinetic study of the effects of morphine on CTL activity in alloimmunized mice is underway. In addition, the involvement of corticosterone in this model system is under analysis. The results would suggest that the acute administration of morphine to mice prior to alloimmunization dramatically suppresses peritoneal CTL activity. Serum corticosterone levels are elevated in the morphine administered animals relative to vehicle-treated controls at the 2 h time point but not 12 h or 120 h following morphine administration. To confirm the role of corticosterone involvement in the morphine-mediated suppression, the corticosterone synthesis inhibitor, cyanoketone is currently under study. It is anticipated that these studies will be concluded by October 1, 1994. The results of this investigation will be directly applicable to the studies involving viral reactivation and the neuroendocrine systems involved. Further studies regarding the role of peripheral and central α-adrenergic pathways in the reactivation of HSV-1 from latency and the effect on the immune system are in progress.

CONCLUSIONS:

We are very encouraged by the results obtained in this first year of investigation. It is clear that the stress models of viral reactivation represent a reliable and definitive means of inducing neuroendocrine changes which modulate immunological function and result in the reappearance of infectious virus in the central and peripheral tissues of the infected host. This reliable and reproducible system will afford us the opportunity of further dissecting the neuroendocrine immunologic system involved in stress-mediated viral reactivation.

The detailed analyses of the neuroendocrine mechanism involved in stress-induced viral reactivation have revealed a role for the classical neuroendocrine mediators such as corticosterone and have indicated that there is a marked and dramatic affect of the neuroendocrine system on the expression of cytokine genes in immunologically competent effector cells. Our investigations of the qualitative and quantitative nature of the neuroendocrine affects on cytokine gene expression and hence the immunologic competence of the infected host, are now in progress. We have already obtained preliminary data indicating that through a complex series of interactions and feedback mechanisms classical neurohormones such as corticosterone, catecholamines, and probably adrenocorticotrophic hormone (ACTH) down-regulate the host immune response through an alteration in cytokine gene expression, particularly affecting the pro-inflammatory cytokines such as IL-1 and IL-6, but as well modulating the expression of

IL-2 and gamma interferon genes permitting viral reactivation to occur.

Future studies will involve an investigation of the affect of externally applied catecholamines and corticosterone antagonists and agonists. These studies will permit us to determine the direct or indirect role of these mediators to prevent viral reactivation in stressed and infected hosts. Additional studies which will better define the role of the neuroendocrine immunologic axis in viral reactivation will focus on the cellular and humoral parameters which are important in the defense against recurrent viral infection. The affect of stress on the activation of the immune defense system during viral reactivation will be further investigated.

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APPENDICES

Chapter 6
Appendix Item 1

HSV-1 LATENTLY-INFECTED MICE DISPLAY AN ALTERED RESPONSE TO STRESS:IMPLICATIONS FOR ANTIVIRAL IMMUNITY

William Halford○, Bryan Gebhardt‡○,and Daniel J.J. Carr○†
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Reactivation of herpes simplex virus type-1 (HSV-1) from latency occurs in response to stress. Stress may trigger recurrent herpetic disease not only by reactivating viral gene expression, but also by suppressing antiviral immunity. To assess the potential interaction between latent HSV-1 infection and the stress response of mice (and in turn the immune response), induction of endocrine and paracrine mediators was compared in four treatment groups: infected/ stressed, infected/ not stressed, uninfected/ stressed, and uninfected/ not stressed. In samples taken 24 hours after a heat/ restraint stressor, differences were observed. Serum corticosterone levels in infected mice were higher than uninfected mice ($p<.05$). Serum concentrations of corticosterone and interleukin-6 (IL-6) in uninfected/ stressed mice were positively correlated ($r=.87$), but were negatively correlated ($r=-.28$) in infected/ stressed mice. This suggests that an underlying difference exists in the regulation of IL-6 and/or corticosterone between latently infected and uninfected animals following exposure to a stressor ($p=.005$). RT-PCR analysis further indicates that IL-6 expression is significantly lower in the trigeminal ganglia (site of latency) of mice latently infected with HSV-1. The results suggest that HSV-1 can alter host regulation of immunomodulatory mediators such as IL-6 and corticosterone. This may be significant in stress-induced immunomodulation during recurrent herpetic disease.

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Chapter 6
Appendix Item 2

MOUSE LYMPHOCYTES EXPRESS AN ORPHAN OPIOID RECEPTOR

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Pharmacologic evidence indicates that lymphocytes respond to opioid agonists, but the expression of opioid receptor mRNA by lymphocytes has not been demonstrated. Using reverse transcription-polymerase chain reaction (RT-PCR), we have amplified and sequenced two-thirds of an orphan opioid receptor message from Balb/c lymphocyte RNA. Oligonucleotide primers were initially made against sequences encoding transmembrane (TM) III and TM V of a δ-opioid receptor, DOR-1, because these regions are highly conserved among opioid receptors. Using these primers, a 382 bp product was obtained from mouse lymphocyte RNA by RT-PCR. This lymphocyte mRNA sequence is identical to an orphan opioid-receptor mRNA recently isolated from mouse brain, except that the amplified lymphocyte sequence contains an 81 base intron which lies within the coding sequence of the second extracellular loop of the opioid receptor. Homologous orphan opioid receptor messages, hORL1 and ROR-C, have recently been isolated and sequenced from human brain and rat brain cDNA libraries, respectively. Primers for the 5′ and 3′ ends of this mRNA have since been used in nested RT-PCR to amplify and sequence 842 bases from the 3′ end of the lymphocyte orphan opioid receptor mRNA. These results indicate that lymphocytes express an opioid receptor which is similar, if not identical, to an orphan opioid receptor identified in cells of the central nervous system.

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MORPHINE SUPPRESSES PERITONEAL AND SPLENIC CTL ACTIVITY IN A DOSE DEPENDENT FASHION IN ALLOIMMUNIZED MICE. **Marc L. Baker[1], Daniel J.J. Carr[1,2], & Bryan M. Gebhardt[1,3].** *[1]LSU Neuroscience Center, Departments of [2]Microbiology, Immunology, Parasitology and [3]Ophthalmology, LSU Medical Center, New Orleans, LA.*

Previous results show chronic exposure to morphine (50 mg/kg s.c., daily) over 11 days in alloimmunized C3H/HeN mice suppresses CTL activity of splenic lymphocytes (SL) and peritoneal lymphocytes (PL). To further these initial studies, a dose response curve using morphine (10.0, 32.0, 56.0, and 100.0 mg/kg, s.c.) and measuring CTL activity was carried out in alloimmunized CD1 mice. Whereas low (10.0 mg/kg) and high (100.0 mg/kg) doses of morphine administered daily over 5 days had no effect on splenic lymphocyte (SL) or peritoneal lymphocyte (PL) CTL activity, intermediate doses (32.0 and 56.0 mg/kg) of morphine significantly suppressed (40-50 %) both SL and PL CTL activity. None of the doses tested had any effect on splenic NK activity. Since previous results that have shown chronic morphine administration induces thymic atrophy, thymocyte populations were evaluated for changes in the percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^+CD8^+$ cells. The results show a marked decrease (50 %) in total thymocytes recovered from animals treated with 32.0 56.0 and 100.0 mg/kg morphine compared to 10.0 mg/kg morphine or vehicle-treated groups. However, the percentage of thymocyte subpopulations in all treatment groups was similar. Similar to the results obtained with the thymocyte populations, there were no detectable differences in subpopulations ($CD4^+$ & $CD8^+$) of SL measuring all treatment groups. Collectively, the reduction in CTL activity of SL and PL populations is not due to changes in the cellular constituents of the organs nor is it due to changes in the overall T cell precursor numbers found in the spleen. It is predicted changes at the effector cell level are ultimately responsible for defects observed in the cytolysis of the CTL population.

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THE FREQUENCY OF EXPOSURE TO MORPHINE DIFFERENTIALLY AFFECTS CTL ACTIVITY IN ALLOIMMUNIZED MICE. **Daniel J.J. Carr[1], Lisa Breeden[1], Gregory W. Carpenter[1], & Bryan M. Gebhardt[2].** *Departments of [1]Microbiology, Immunology, Parasitology and [2]Ophthalmology, LSU Medical Center, New Orleans, LA.*

Previous results show that chronic exposure to morphine (50 mg/kg s.c., daily) over 11 days in alloimmunized C3H/HeN mice suppresses CTL activity of splenic lymphocytes (SL) and peritoneal lymphocytes (PL). To further determine the time-dependency of this observation, C3H/HeN mice were exposed either acutely (one time 2 hours prior to alloimmunization) or subchronically (daily over a 120 hour time period) with morphine (50 mg/kg, s.c.) in C3H/HeN mice immunized with 1 X 10^7 C57BL/6 SL. Five days after alloimmunization, the mice were sacrificed and SL and PL were assessed for CTL activity using EL-4 lymphoma cells in a 4-hr ^{51}Cr-release microcytotoxicity assay. Whereas subchronic exposure to morphine had no effect on PL or SL CTL activity, acute morphine exposure significantly suppressed PL CTL activity. Specifically, PLs from vehicle-treated mice displayed 44.8 ± 7.9 lytic units (LU) compared to 20.0 ± 6.0 LU from PLs of acute morphine-treated mice. Since morphine administration *in vivo* has been linked to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, serum corticosterone levels were measured at 2, 12, and 120 hours post morphine administration. The results show that serum corticosterone levels are elevated [$F(1,9) = 12.5533$, $p < .01$) in the morphine-treated mice (901 ± 45 ng/ml, n = 5) compared to levels in vehicle-treated animals (505 ± 102 ng/ml) at the 2 hr time point. Measurements of serum corticosterone at the other time points are currently in progess. Assessment of the effects of morphine on *in vitro* generation of CTLs was also determined. Morphine (10^{-5} - 10^{-11} M) added (at the time of initiation of culture or daily over the 5 day incubation period) to one-way mixed lymphocyte cultures (C3H/HeN x C57BL/6J spleen cells) was found to have no effect on the generation of CTLs. Taken together, the administration of morphine induces a time-dependent suppression of CTL activity in alloimmunized mice through an indirect pathway.

(This work was supported by a grant from the Department of Defense).

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Chapter 6
Appendix Item 5

Cellular Mechanisms Involved in Morphine-Mediated Suppression of CTL Activity[1]

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INTRODUCTION

Cellular immunity including MHC-unrestricted natural killer (NK) cells and antigen-specific, class I MHC-restricted cytotoxic T lymphocytes (CTLs) plays a central role in monitoring viral infections and tumor growth (1). NK activity has been shown to be modified by opioid compounds both *in vitro* and *in vivo*. The addition of endogenous opioid peptides (e.g., ß-endorphin or [met]-enkephalin) to 4-hr ^{51}Cr-release NK microcytotoxicity assays has been shown to augment NK activity; this augmentation is naloxone-sensitive (2). However, the acute administration of opioid drugs (e.g., morphine or fentanyl) in mice has been shown to suppress splenic NK activity through a naltrexone-sensitive mechanism (3-5). This suppression involves opioid receptors located in the periaqueductal gray matter of the mesencephalon (6). Pretreatment of the mice with the α-adrenoceptor antagonists phentolamine or prazocin blocks morphine-mediated suppression of splenic NK activity implicating α-adrenergic receptor involvement (7). Preadministration of mice with phentolamine (general α-adrenoceptor antagonist) but not doxazosin (peripheral-acting α-adrenergic receptor antagonist) inhibits morphine-mediated suppression of splenic NK activity further implicating central (brain) rather than peripheral α-adrenergic involvement (8). Alternatively, other neuroendocrine hormones may be utilized distal to the brain ultimately influencing NK effector cells. Specifically, splenic serotonin levels are elevated following acute morphine administration and such increases can be blocked by pretreating animals with phentolamine (8). These results suggest serotonin might be solicited by adrenergic processes ultimately resulting in suppression of splenic NK activity. Consistent with this notion, a recent study revealed serotonin suppressed NK activity in whole blood and such effects could be reversed with interferon-α (9). Certainly, this is a complicated issue involving many mediators which may have direct or indirect effects on the NK cells.

At the cellular level, NK effector cells from acute morphine-treated mice have a reduced capacity to form conjugates with target cells (8). In addition, of those cells which can form conjugates with their targets, a reduced number initiate lysis of targets relative to NK-enriched effector cells from vehicle-treated mice (8). These results are consistent with the observation showing the reduction in splenic NK activity is not due to a redistribution of NK effector cells ($NK1.1^+2B4^+$) from the spleen (8). Taken together, the data suggest acute morphine administration modifies cellular machinery involved in the "lethal hit" of the NK effector cells with

the spleen (8). Taken together, the data suggest acute morphine administration modifies cellular machinery involved in the "lethal hit" of the NK effector cells with their targets. Currently, tyrosine kinase activity is under investigation since previous studies indicate a correlation between tyrosine kinase activity and NK-mediated cytolysis of target cells (10,11).

Contrary to the effects of opioids on *in vitro* and *in vivo* NK activity, less information is available concerning the action of opioids on CTL activity. The generation of CTLs in *in vitro* one-way mixed lymphocyte cultures (MLCs) has previously been shown to be augmented by ß-endorphin and [met]-enkephalin in a naloxone-reversible manner (12). However, the *in vivo* effect of opioids on CTL generation or activity is not known. Consequently, a study was undertaken to assess the effect of chronic morphine exposure on CTL activity in alloimmunized mice.

CHRONIC MORPHINE EXPOSURE SUPPRESSES CTL ACTIVITY IN ALLOIMMUNIZED MICE

A study was initiated to investigate chronic (11 days) morphine exposure on NK and CTL activity in alloimmunized mice. Specifically, C3H/HeN (H-2^k haplotype) mice were administered with morphine (50 mg/kg, s.c.) two hours prior to receiving 1 X 10^7 C57BL/6 (H-2^b) spleen cells, i.p.. Following the immunization, mice received vehicle or morphine (50 mg/kg) daily for an additional 6 days. On day 7, mice were reimmunized with 1 X 10^7 C57BL/6 spleen cells, i.p two hours after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional three days. On day 11, the mice were sacrificed and peritoneal exudate leukocytes (PL) and splenic lymphocytes (SL) were assessed for NK and CTL activity. It is our opinion that this treatment regimen more closely reflects the habits of an opioid abuser who must continually administer drug in order to avoid the effects of withdrawal. Chronic exposure to morphine significantly suppressed SL and PL CTL activity as determined using ^{51}Cr-labeled EL-4 cells (H-2^b) in a 4-hour microcytotoxicity assay (13). The SL from either vehicle- or morphine-treated groups showed no measurable cytolysis against third party targets (P815, H-2^d) indicating the antigen-specificity of the target cells. Similarly, SL from non-immunized mice showed no measurable cytolytic activity against ^{51}Cr-labeled EL-4 cells. CD4- and CD8-enrichment studies showed the CTL effector cells were phenotypically-defined as $CD4^-CD8^+$.

Opioid receptor antagonists were used to define the opioid specificity of morphine-mediated suppression of SL and PL CTL activity. Previous studies utilized ß-funaltrexamine (ß-FNA, μ-selective opioid receptor antagonist), naltrindole (δ-selective opioid receptor antagonist), norbinaltorphimine (κ-selective opioid receptor

antagonist), and naloxonazine (μ_1-selective opioid receptor antagonist) to identify the involvement of μ_2-opioid receptors in the suppression of splenic NK activity following acute morphine administration (7). In the present investigation, ß-FNA and (E)-7-benzylidine-7-dihydronaltrexone (BNTX, δ-selective opioid receptor antagonist) (14) were used to determine μ versus δ opioid receptor involvement. The pretreatment of mice with ß-FNA (40 mg/kg every 72 hours and 18-24 hours prior to receiving morphine) effectively antagonized the suppression of PL and SL CTL activity elicited by chronic morphine treatment (Carpenter & Carr, submitted). The pretreatment of mice with BNTX did not block morphine-mediated suppression of SL CTL activity (Carr & Carpenter, submitted). However, the mice pretreated with BNTX antagonized morphine-mediated suppression of PL CTL activity (Carr & Carpenter, submitted). Taken together, the results indicate morphine-mediated suppression of SL CTL activity utilizes μ opioid receptors while the type(s) of opioid receptors associated with morphine-mediated suppression of PL CTL activity has not been resolved.

The possibility that morphine might have a direct effect on CTL generation or activity was investigated *in vitro*. Specifically, morphine (10^{-5} - 10^{-11} M) added either during the initiation of culture or daily throughout the one-way MLC showed no effect on the generation of CTLs suggesting morphine does not interact directly with lymphocytes (presumably through opioid receptors) in modifying CTL activity (unpublished observation).

To define the biological significance of morphine-mediated suppression of CTL activity, mice were infected with the LD_{50} of the McKrae strain of herpes simplex virus in the hind footpad and subsequently administered vehicle or morphine (50 mg/kg) daily over a 21 day period. The results showed chronic morphine-treated animals died earlier and in greater numbers compared with the vehicle-treated controls (13). Consequently, the assertion that morphine might be a co-factor in the acquisition of viral infections including AIDS (15) is supported by this data.

To identify the cellular mechanisms linked to the recognition and lysis of the target cell which might be altered following morphine exposure *in vivo*, a study was undertaken to assess target recognition and selective pathways associated with some of the lytic processes functional during the "lethal hit" of the target by effector cells from vehicle- and chronic morphine-treated mice.

CHRONIC MORPHINE TREATMENT SUPPRESSES GRANULATION AND cAMP RESPONSES TO ALLOANTIGEN BY CTL EFFECTOR CELLS

The recognition of targets by effector cells and subsequent activation of effector cells prior to lysis of targets involves a complex intracellular signalling linguistics (Fig. 1). Initially, the $CD8^+$ effector cell recognizes the peptide antigen association

with class I MHC expressed on the surface of the target cell (in this case, the EL-4 [H-2^b] lymphoma) by the T cell receptor (TCR). In addition, the CD8 molecule on the effector cell interacts with the class I molecule of the target increasing the stability of the TCR-MHC class I interaction. Furthermore, the LFA-1 (CD11a) antigen on the effector cell interacts with the integrin counterpart (CD54) of the target further increasing the stability of the effector/target interaction. The interaction of CD11a with CD54 may result in the phosphorylation (serine residue of the ß-chain, CD18) of the cytoplasmic domain of CD11a although the result of this phosphorylation has not been conclusively determined. Another surface antigen on the effector cells CD45 may play an extremely important role in cell activation. The phosphotyrosine phosphatase activity associated with the CD45 molecule may act to dephosphorylate the kinase domain of $p56^{lck}$ and $p53^{fyn}$ src-protein tyrosine kinases (16). Since the $p56^{lck}$ has a amino-terminal cysteine motif which can interact with the CD8 cytoplasmic tail, it places this tyrosine kinase in close proximity to the TCR-CD3 complex. Consequently, the SH2 domain of the $p56^{lck}$ or $p53^{fyn}$ can associate with the phosphorylated Γ or zeta chains of the CD3 complex. A syk-related tyrosine kinase ZAP-70 driven by the phosphorylation of the zeta chain of the CD3 molecule may be involved in activation of phospholipase $C_{γ1}$ ultimately resulting in the hydrolysis of phosphatidylinositol 4,5 bisphosphate generating inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). DAG has been shown to transiently stimulate protein kinase C (PKC) resulting in serine/threonine phosphorylation leading to the activation of the effector cell. In addition, IP_3 generation drives the mobilization of Ca^{2+} which through either cAMP-dependent or independent mechanisms increases the translocation of granules to the membrane for delivery to the target. Following the initial recognition of the target, one or more of the intracellular signalling pathways described above could be altered in effector cells from the morphine-treated mice. Previous work has described the suppression of CD2 expression (17) and Ca^{2+} mobilization (18) in the presence of morphine. Since CD2 has been implicated in the regulation of CD45R (16) and Ca^{2+} mobilization is linked to granzyme A-mediated cytolysis of targets by effectors (19), we have explored some of these pathways for possible modification following morphine exposure.

@Figure = Figure 1. Signalling transduction pathways associated with $CD8^+$ activation following target cell recognition.

There are currently two proposed mechanisms of lymphocyte-driven cytotoxicity: (i) non-secretory, a receptor-mediated triggering of apoptosis and (ii) secretory, a membranolytic mechanism (19). CTL-mediated cytotoxicity includes both mechanisms, a Ca^{2+}-dependent membranolytic pathway (20) and an apoptotic receptor-driven pathway which may not involve extracellular Ca^{2+} (19). To determine which mechanism is operational in our system, splenic CTLs generated in the above treatment regimen were assessed for cytolysis of targets (EL-4) in the presence of the Ca^{2+}-chelating agent, ethyleneglycol-bis-(ß-aminoethylether)N,N'-

tetra-acetic acid (EGTA). Splenic CTLs from either chronic morphine- or vehicle-treated mice did not elicit the "lethal hit" in the presence of EGTA as measured by ^{51}Cr release (Carr & Carpenter, submitted) indicating a Ca^{2+}-dependent cytolytic process. The Ca^{2+}-dependent, secretory membranolytic mechanism of target cell lysis involves the release of granules, granzymes, and perforin which function to initiate pore formation in the target cell membrane ultimately resulting in internal disintegration and DNA fragmentation (19). Constituents in granzymes include serine esterases which have been implicated in the cytolytic process of CTLs and NK cells (19). Accordingly, we compared the levels of serine esterases in response to alloantigen from SL of vehicle- and chronic morphine-treated mice. The results show the percent of serine esterase release was lower in the SL taken from chronic morphine-treated animals compared to serine esterase release from vehicle-treated controls (13). In addition, there was a significant decrease in the total serine esterase content in SL obtained from the chronic morphine-treated mice. These results suggest one means of morphine-mediated suppression of CTL activity is by decreasing the levels and release of cytolytic-associated enzymes by effector cells. Another intracellular event implicated in the degranulation of effector cells is the rise in cAMP (21). In addition, cAMP has been associated with detachment from the target cell as well as a signal responsible for the initiation of recycling by CTLs for subsequent lytic function (21). Consequently, we investigated cAMP levels by $CD8^+$-enriched effector cells following alloantigen exposure *in vitro*. The results show $CD8^+$-enriched effector cells from the chronic morphine-treated mice do not respond with an elevation in cAMP levels following alloantigen exposure compared to cells from vehicle-treated mice. Taken together, the data suggest CTLs from chronic morphine-treated mice have an aberrant intracellular signalling transduction pathway which results in a decrease in serine esterase release ultimately resulting in a reduction in CTL activity.

Previous results show NK-enriched effector cells taken from acute morphine-treated mice conjugate less effectively with their targets compared to NK effector cells from vehicle-treated controls (8). Therefore, we investigated CTL effector cells from chronic morphine- and vehicle-treated groups for their ability to conjugate with targets. The results show CTLs from either morphine- or vehicle-treated mice conjugate equally well with their targets (13). In addition, the expression of CD11a on $CD8^+$ effector cells from vehicle- and morphine-treated mice was at similar levels which correlates with the conjugate studies. Collectively, the data seem to indicate the number of CTLs generated over the 11 day treatment regimen with either vehicle or morphine does not differ. Instead, the suppression in measurable CTL activity is due to a defect in the effector cell-associated lytic mechanism(s).

CHRONIC MORPHINE TREATMENT ALTERS HYPOTHALAMIC PITUITARY ADRENAL AXIS HORMONE LEVELS

Previous studies have shown hypothalamic pituitary adrenal (HPA) axis involvement in immunomodulation following short term exposure to morphine (22-24). Specifically, the administration of morphine elicits a stress response with a subsequent increase in serum corticosterone levels. Based on these results, a study was conducted to measure corticosterone levels in the serum of vehicle- and chronic morphine-treated mice. Interestingly, the results showed serum corticosterone levels were lower in the chronic morphine-treated mice relative to vehicle-treated controls (13). However, these measurements were taken at the end of the treatment regimen on day 11 following the sacrifice of the animals and do not necessarily indicate the potential relationship between the hormone and morphine-mediated suppression of CTL activity. It is tempting to speculate that these levels reflect the tolerance of the mice to morphine since the characteristic straub tail associated with morphine administration was less apparent towards the end of the treatment regimen (days 9 and 10, unpublished observation).

Still another possibility of morphine-mediated immunosuppression might reside in the lymphocyte-derived, proopiomelanocortin (POMC) peptide hormone levels. Specifically, the POMC-derived hormone, ß-endorphin has previously been shown to augment CTL activity (12). We hypothesized that chronic exposure to morphine may reduce the level of expression of the POMC transcript encoding ß-endorphin and hence, reduce ß-endorphin levels. By using reverse transcription-polymerase chain reaction and oligonucleotide primers selective for exon 3 of POMC (encodes ß-endorphin), the results showed no differences in the expression of POMC by SL obtained from either treated group of animals (13). Specifically, SL from 3 of 13 vehicle-treated mice were positive for exon 3 POMC mRNA while the lymphocytes from 6 of 14 chronic morphine-treated mice were also positive. Consequently, the levels of POMC do not correlate with morphine-mediated immunosuppression and probably play no role in this model system.

@Figure = Figure 2. Immunomodulation via opioids. Proposed mechanisms.

SUMMARY

Based on a plethora of data from many laboratories, we have proposed the following mechanisms by which morphine alters immune homeostasis and immunocompetence *in vivo* (Fig. 2). Specifically, the administration of morphine subcutaneously via routing through blood interacts directly with opioid receptors on cells of the immune system or on receptors within the central nervous system. Although there is currently no evidence to support the direct involvement of morphine on lymphocyte opioid receptors, *in vitro* studies show the existence of functional, naloxone-sensitive opioid receptors (25). In addition, pharmacological and biochemical characterization of lymphocyte opioid receptors has been shown to be consistent in many instances, with the profile of neural-derived opioid

receptors (25-27). Finally, recent molecular studies using oligonucleotide primers specific for the δ-class opioid receptor cloned from NG-108-15 cells (28) have been used in reverse transcription-polymerase chain reactions to generate a 400 bp product in SL which has 100% sequence homology with a published opioid receptor cloned from a brain library (Halford, Gebhardt, and Carr, unpublished observation). However, future studies are necessary to establish the role of lymphocyte opioid receptors following the *in vivo* administration of opioids (e.g. fentanyl, methadone, and morphine).

Since the administration of morphine subcutaneously appears to predominately interact with brain opioid receptors (3) located in the mesencephalon (5), other neuroendocrine systems become candidates for activation and subsequent direct modulation of immune function: (i) the HPA axis and (ii) the sympathetic nervous system (SNS). The activation of the HPA axis through the release of corticotropin releasing hormone elicits the production of adrenocorticotropin hormone (ACTH) by corticotrophs of the anterior pituitary which in turn travels through the blood to the adrenals and elicits the production of corticotropin. Corticotropin can then act on lymphocytes resulting in suppression of selective immune parameters predominately T cell-mediated (29). The activation of the SNS by morphine does modify immune responsivity through the "hard-wiring" of immune organs (thymus, spleen, bone marrow, and lymph nodes) (30). Specifically, studies have shown SNS involvement in morphine-mediated suppression of lymphocyte proliferation as well as interleukin (IL)-2, IL-4, and interferon-Γ production (31), and natural killer activity (7). Whereas central α-adrenoceptors are involved in morphine-mediated suppression of splenic NK activity (8), peripheral ß-adrenoceptors are involved in morphine-mediated suppression of lymphocyte proliferation and cytokine production (32). Finally, the consideration of cytokines including neuroendocrine peptide hormones produced by immune cells in response to neural stimulation in the lymphoid organ following morphine administration must also be taken into account. Whether these products or blood borne neuroendocrine hormones including catecholamines and corticosterone ultimately are responsible in the suppression of immunocompetence following morphine administration remains a question. Based on the studies presented herein, we have concluded the SNS and HPA are the predominate sources involved in morphine-mediated effects on the immune system.

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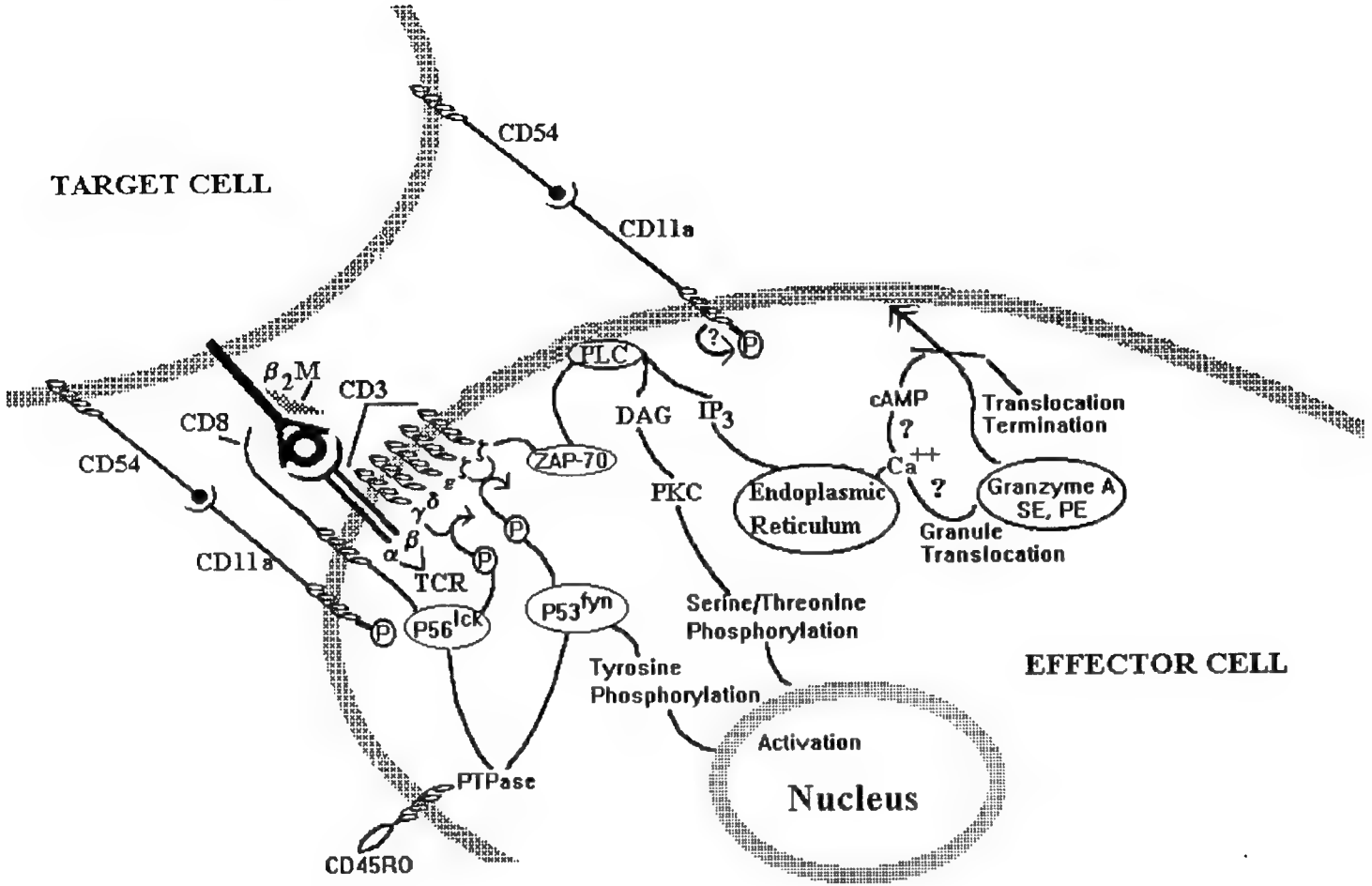
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Fig 1

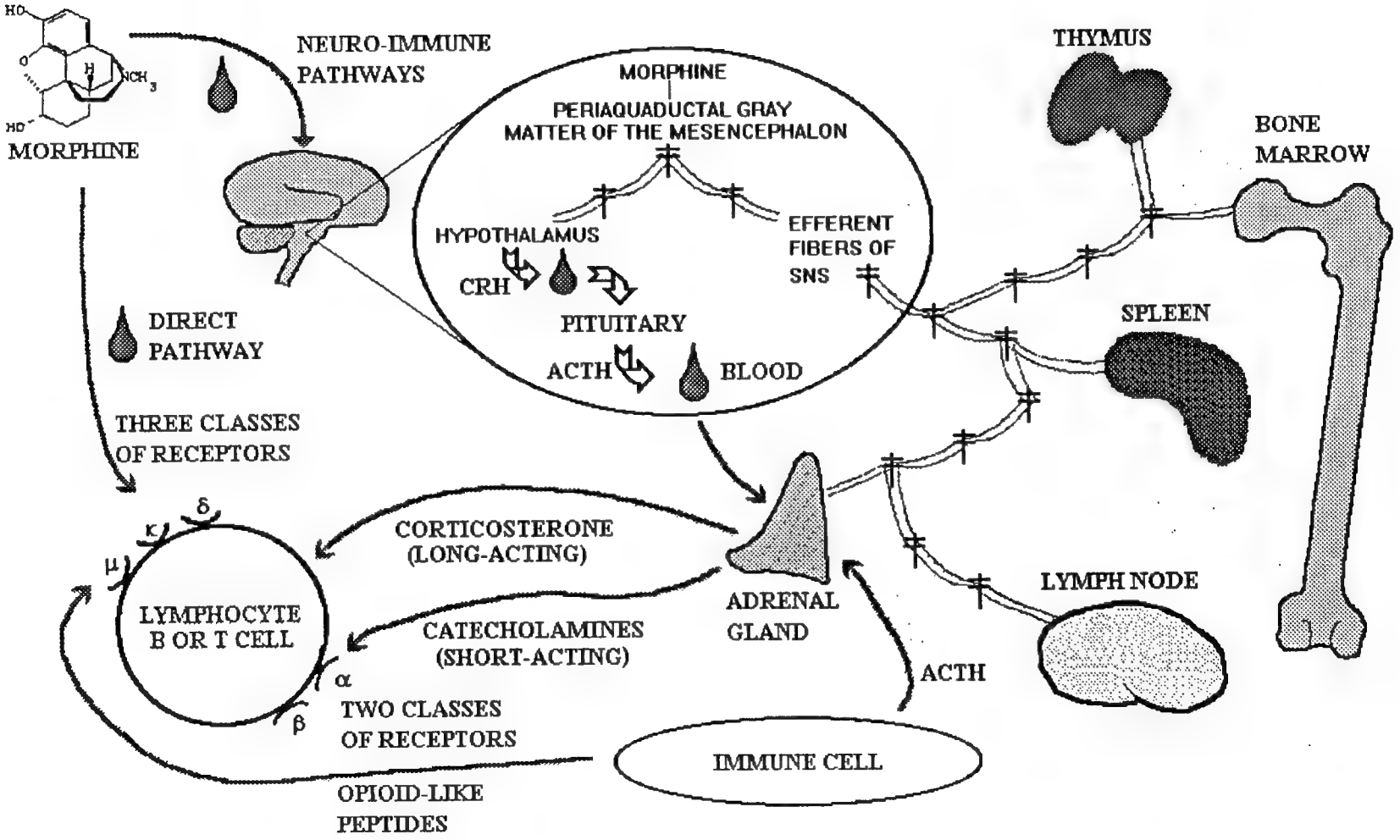
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Fig 2

Submitted - Immunopharmacology

Chapter 6
Appendix Item 6

Pretreatment With ß-Funaltrexamine Blocks Morphine-Mediated Suppression of CTL Activity in Alloimmunized Mice

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ABSTRACT

The effect of prolonged exposure to morphine on cytotoxic T lymphocytes (CTL) and splenic natural killer (NK) activity was investigated. Daily administration of morphine (50.0 mg/kg, s.c.) to alloimmunized mice for 11 days resulted in a significant decrease (25-50%) in peritoneal and splenic CTL activity but not splenic NK activity. Effector cell enrichment studies indicate the CTLs are $CD8^+$ $CD4^-$. Chronic morphine treatment increased the percentage (25-30%) of $CD3^+CD4^+$, and $CD8^+$ but not Ig^+ cells in the spleen relative to saline-treated controls. Pretreatment of mice with the μ-selective antagonist, ß-funaltrexamine blocked morphine-mediated suppression of splenic and peritoneal CTL activity as well as the increase in $CD3^+CD4^+$ and $CD8^+$ splenic lymphocytes. These results indicate the generation of CTLs in vivo is sensitive to chronic morphine exposure implicating opiates as important co-factors in viral infections through modulation of cell-mediated immunity.

key words: cytotoxic T lymphocyte, morphine, natural killer activity, and ß-funaltrexamine.

Abbreviations: NK, natural killer; CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; HPA, hypothalamic-pituitary adrenal; PL, peritoneal exudate leukocytes; SL, splenic lymphocytes; ß-FNA, ß-funaltrexamine; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; PWM, pokeweed mitogen; TdR, thymidine deoxyribonucleic acid; DMSO, dimethyl sulfoxide; FACS, fluorescence activated cell sorter; PBS, phosphate buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Ig, immunoglobulin.

INTRODUCTION

The abuse of opioid compounds is predicted to be a major co-factor in the acquisition and spread of human immunodeficiency virus (HIV)-1 (Donahoe, 1992) due to the immunosuppressive side-effects of such drugs. Specifically, morphine has been shown to suppress picryl chloride-induced delayed-type hypersensitivity (Bryant & Roudebush, 1990), splenic NK activity (Shavit et. al., 1984; Weber & Pert, 1989), primary antibody production (Pruett et. al., 1992; Bussiere et. al., 1993), and resistance to viral infections (Lorenzo et. al., 1987; Starec et. al., 1991). Similarly, immunocompetence is compromised in human heroin users (Novick et. al., 1989; DeShavo et. al., 1989; Klimas et. al., 1991) resulting in greater susceptibility to infectious agents (Dismukes et. al., 1968) including HIV-1 (Hubbard et. al., 1988). The acute administration of morphine suppresses NK activity through the activation of α-adrenergic pathways (Carr et. al., 1993; Carr et. al., 1994a) while chronic morphine administration activates the hypothalamic-pituitary adrenal (HPA) axis resulting in elevated levels of corticosteroids which are, in part, immunosuppressive (Bryant et. al., 1991). However, the relationship between the HPA axis and other neuroendocrine pathways relative to opioid-induced modulation of immune homeostasis (specifically NK and CTL activity) is still largely unknown.

In the present study, the effects of chronic administration of morphine on CTL activity in mice was investigated. Previous studies have shown the endogenous opioid peptides [Met]-enkephalin and ß-endorphin augment the generation of CTLs in one-way MLCs *in*

vitro through a naloxone-sensitive pathway (Carr & Klimpel, 1986). Since opioid abusers show an increased susceptibility to viral infections (Dismukes et. al., 1968; Hubbard et. al., 1988), an investigation assessing the immunomodulatory characteristics of morphine on CTL activity was undertaken.

MATERIALS & METHODS

Mice and tumor lines

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) and C3H/HeN (Harlan-Sprague Dawley, Indianapolis, IN) mice were housed in groups of 6-10 per cage and maintained on a 12 h light/dark cycle. Access to water and food (Purina Mouse Chow) was available *ad lib*. The YAC-1 mouse lymphoma cell line, P815 mastocytoma cell line, and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD); the cells have been maintained in culture by biweekly passage over a five month period.

Morphine treatment regimen

A dose-response study has established that 50.0 mg/kg of morphine s.c. results in maximal suppression of cytolytic activity (Carr et. al., 1994b). Consequently, this dose was used in all experiments.

C3H/HeN mice (n = 8/group) were administered the μ-opioid receptor alkylating agent ß-funaltrexamine (ß-FNA, 40.0 mg/kg, s.c.) or vehicle 18-24 h prior to receiving morphine (50.0 mg/kg). Two hours following drug or vehicle administration, mice received 1 X 10^7 C57BL/6 spleen cells, i.p. Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On day 7, mice were re-immunized with 1 X 10^7 C57BL/6 spleen cells, i.p. 2 hours after the administration of vehicle or morphine. In addition,

mice received vehicle or ß-FNA (40.0 mg/kg, s.c.) every 72 h up through the 10 day incubation period. This time period corresponds to opioid receptor turnover as a result of occupancy of receptors by ß-FNA (Dr. Dennis Paul, personal communication). On day 11, the mice were sacrificed and splenic lymphocytes (SL) and peritoneal exudate leukocytes (PL) were collected and assayed for mitogen responsiveness and CTL and NK activity. The phenotypes of the spleen cells of vehicle- and drug-treated mice were determined by flow cytometry.

SL, and PL preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hank's balanced salt solution (HBSS). Cells were collected by recovery of peritoneal lavage fluid through a 20 gauge needle and 10 ml syringe. Spleens were removed and cell suspensions were prepared by mechanical dispersion. SL and PL were washed with HBSS (250 x g, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4CL; the cells were subsequently washed with HBSS (250 x g, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybri-max (Sigma Chemical Co., St. Louis, MO) antibiotic/antimycotic solution (complete media). Cells were counted and examined for viability via trypan blue exclusion dye.

Mitogen-induced proliferation assay

SL from C3H/HeN mice (2 X 10^5 cells/well) in 100 μl of complete media were placed in 96-well microtiter plates (Costar, Cambridge, MA). One hundred microliters of complete

media containing 100 ng pokeweed mitogen (PWM) was added to the wells. Spleen cells were cultured in 5% CO_2 at 37° C for 48 h. After 48 h, 200 nCi [^{3}H]thymidine deoxyribonucleic acid (TdR) in HBSS (10 μl) was added to each well and the cells cultured an additional 12 h. The cells then were harvested on glass fiber filter strips using a multiple-well harvester (Cambridge Technologies, Watertown, MA). Filters were placed in scintillation vials containing 6.0 ml of Cytoscint liquid scintillation cocktail (ICN, Irvine, CA) and allowed to equilibrate 18-24 h. The incorporation of [^{3}H]TdR was determined by liquid scintillation counting using a Beckman LS9800. The mitogenic response of spleen cells from each animal was assayed in quadruplicate. Incorporation of [^{3}H]TdR by cells cultured in the absence of PWM was less than 5% of that obtained in maximally stimulated cultures.

<u>^{51}Cr-release cytolytic assay</u>

SL and PL CTL activity was assayed using a 4 h microcytotoxicity assay with ^{51}Cr-labeled EL-4 cells (H-2^b) as targets. Between 5 X 10^4 and 160 X 10^4 effector cells were mixed with 1 X 10^4 target cells in conical 96-well microtiter plates (Costar, Cambridge, MA) in a reaction volume of 0.2 ml of complete media. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100 μl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percent cytolytic activity = [(experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release - spontaneous ^{51}Cr release)] X 100 where "spontaneous" refers to ^{51}Cr release by target cells in the absence

of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr content in the supernates of 10^4 target cells incubated at 37°C in a 5% CO_2 atmosphere in the presence of 0.1% t-octylphenoxypolyethoxyethanol in complete medium or measuring the ^{51}Cr content in 10^4 ^{51}Cr-labeled target cells. Spontaneous release was consistently between 10-15%. Each effector to target cell ratio (100:1, 50:1, 25:1, and 12:1 for SL and 50:1, 25:1, 12:1, and 6:1 for PL) was measured in triplicate/animal. One lytic unit (LU) is defined as the number of splenic lymphocytes, peritoneal lymphocytes or enriched effector cells able to lyse 20% of the target cells (YAC-1 or EL-4 target cells) and this unit is expressed per 10^7 total cells. To determine antigen specificity for the CTL assay, P815 (H-2^d) mastocytoma cells were ^{51}Cr-labeled and used as targets in the 4 h microcytotoxicity assay.

CD4$^+$ and CD8$^+$lymphocyte enrichment

Mouse T cell subset enrichment column kits (R & D Systems, Minneapolis, MN) were prepared as suggested by the manufacturer. SL from the saline-treated group were pooled as were SL from the morphine treatment group and separately applied to CD4 and CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets in the ^{51}Cr release cytolytic assay.

Fluorescence activated cell sorter (FACS) analysis of SL subpopulations

SL (1 X 10^6 cells/condition) obtained from the vehicle- and drug-treated groups of mice were collected and washed in 1.0 ml of phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.05 M NaN_3. SL were resuspended in 0.05 ml PBS-BSA plus NaN_3 containing 30 μg of rat IgG_{2b} (isotypic control, Zymed, South San Francisco, CA) and incubated on ice for 10 min. Subsequently, antibody to CD3, CD4, and/or CD8 (rat IgG_{2b}, Gibco BRL, Gaithersburg, MD) conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or α-immunoglobulin [Ig, heavy & light chain specific, $F(ab)'_2$] conjugated with FITC (Boehringer Mannheim, Indianapolis, IN) was added for a final volume of 0.1 ml (using PBS-BSA plus NaN_3). The labeled cells were allowed to incubate for 30 min on ice in the dark. The cells were washed with ice-cold PBS, fixed with 1% paraformaldehyde, and analyzed by FACS for the percentage of stained cells in the cell population. Light scatter was collected at 488 nm and the emitted light which passed through a long pass filter was analyzed at 525 nm (FITC) or 575 nm (PE) on a Coulter Elite FACS (Coulter, Hialeah, FL). Five thousand gated events were analyzed per sample.

Reagents

Morphine sulfate and ß-FNA were generously provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). These drugs were initially dissolved in DMSO and diluted with HBSS to a concentration containing 10-25% DMSO. A volume of 100 μl of this solution containing the drug at the appropriate

concentration was delivered to each mouse. Vehicle consisted of 10-25% DMSO in HBSS.

Statistics

One-way ANOVA (Randomized, block design) was used together with Scheffe or Tukey's post hoc multiple comparisons test to determine significance ($p \leq .05$) between saline- and drug-treated groups. This statistical package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

Chronic morphine exposure suppresses CTL activity.

Mice treated with morphine for 11 days exhibited significantly less SL CTL activity compared to vehicle-treated controls (Fig. 1). PL CTL activity was also significantly lower in the chronic morphine-treated mice compared to vehicle-treated controls (Fig. 2). However, both vehicle- and chronic morphine-treated mice showed similar levels of splenic NK activity (Fig. 3). SL from chronic morphine- and vehicle-treated mice were also assayed for lysis of a histoincompatible target. Neither population of SL showed any measurable cytolytic activity against ^{51}Cr-labeled P815 cells (data not shown). In addition, SL from unprimed (non-immunized) mice had no measurable CTL activity to the ^{51}Cr-labeled EL-4 targets (data not shown).

Using cell separation techniques, the SL CTL derived from vehicle- and drug-treated mice (Fig. 4) were found to be $CD8^+$ (Table I). $CD8^+$-enriched effector cells were also tested against the NK-sensitive target YAC-1 and found not to lyse these cells indicating the antigen-specificity of the enriched effector cells (data not shown). Moreover, enrichment enhanced the difference in CTL activity between SL obtained from vehicle- and chronic morphine-treated mice (Table I).

ß-FNA attenuates opioid-induced suppression of SL and PL CTL activity in chronic morphine-treated mice.

To determine opioid receptor involvement in morphine-mediated suppression of CTL activity, studies were carried out using the μ-selective opioid antagonist ß-FNA in morphine-treated animals. Pretreatment of mice with ß-FNA completely blocked morphine-induced suppression of SL (Fig. 1) and PL (Fig. 2) CTL activity. ß-FNA alone had no effect on SL (Fig. 1) or PL (Fig. 2) CTL activity. ß-FNA alone nor in combination with morphine had any effect on splenic NK activity (Fig. 3).

ß-FNA blocks morphine-induced increases in the percentage of $CD4^+$ and $CD8^+$ splenic lymphocytes.

Since morphine decreased the SL and PL CTL activity and splenic effector cells mediating antigen-specific CTL activity were defined as $CD4^-CD8^+$, morphine exposure might modify the number of lymphocytes in the spleen. Studies were undertaken to assess T_{helper} ($CD3^+CD4^+$), $T_{cytotoxic}$ ($CD8^+$) and B (Ig^+) splenic and peritoneal lymphocyte populations from vehicle and chronic morphine-treated mice in the presence and absence of ß-FNA. Phenotypic analysis of SL populations revealed an increase in the percentage of $CD3^+CD4^+$ and $CD8^+$ but not Ig^+ cells in the chronic morphine-treated mice (Fig. 5). Pretreatment of mice with ß-FNA partially blocked the effects of morphine on the percentage shifts in the SL population (Fig. 5) although ß-FNA alone had no effect.

Changes in the percentage of lymphocyte populations in the spleen may also alter lymphocyte responsivity to antigen (as shown in the CTL activity) or mitogen through the absence of appropriate cytokines necessary to drive lymphocyte growth and differentiation. Accordingly, SL from the treated groups of animals were also evaluated for proliferation to PWM. SL from chronic morphine-treated mice showed a significant increase in response to PWM compared to splenocytes from saline-treated controls (Table II). Pretreatment of mice with ß-FNA partially antagonized the effect of morphine. Pretreatment with ß-FNA alone had no appreciable effect on PWM-stimulated SL proliferation (Table II).

DISCUSSION

In the present study, chronic exposure to morphine resulted in a lower response to alloimmunization as reflected by CTL activity. Since the reduced CTL activity was evident in both PL and SL populations, we speculated that a systemic pathway is involved. The results of previous studies indicate that 72 h exposure to morphine activates the HPA axis resulting in the elevation in serum corticosterone levels (Bryant et. al., 1991). Endogenous corticosterone is selectively immunosuppressive (Stein & Miller, 1993) and may be partly responsible for the effects seen in the present study. However, recent data may indicate otherwise. Specifically, corticosterone levels have been measured in the chronic (11 day) morphine-treated mice following the sacrifice of the animals and found to be reduced in comparison to the levels from vehicle-treated mice (Carpenter et. al., 1994). However, this observation does not rule out the role elevated levels of corticosterone may have earlier in the immune response as indicated by others (Bryant et. al. 1987; Bryant et. al., 1990; Bryant et. al., 1991; Fuchs & Pruett, 1993).

Adrenergic pathways have also previously been shown to be involved in morphine-induced immunosuppression of SL (Carr et. al., 1993, Fecho et. al., 1993). Interestingly, the immunosuppression following acute morphine administration is compartment specific (Bayer et. al., 1990; Baddley et. al., 1993; Lysle et. al., 1993). The compartmentalized nature of morphine-mediated immunomodulation may in part lie with the neuroendocrine systems innervating the immune organ (Felten et. al., 1985) as well as the state of activation of the lymphocytes. Specifically, although previous work indicates

lymphocytes possess μ-type opioid binding sites (Madden et. al., 1987; Radulescu et. al., 1991), recent studies indicate activation upregulates the expression of the morphine-sensitive binding site (Roy et. al., 1992). However, morphine (10^{-5} - 10^{-11} M) effects on the generation of CTLs in one-way MLCs in vitro have resulted in no discernable differences to vehicle-treated controls (unpublished observation); this observation indicates the absence of a direct effect of morphine on lymphocytes. Moreover, a previous study showed morphine-mediated immunoregulation did not correlate with circulating levels of the drug (Bryant et. al., 1988). Collectively, these results suggest morphine-mediated immunomodulation following acute or chronic application of drug in vivo acts in part through neuroendocrine pathways other than the HPA axis as most recently illustrated (Hernandez et. al., 1993; Carr et. al., 1994a).

The pretreatment of mice with the irreversible μ-selective opioid receptor antagonist, ß-FNA (Ward et. al., 1982), effectively blocked the suppression of PL CTL activity precipitated by chronic morphine treatment. Similar findings have also been reported for splenic NK activity following acute morphine administration (Band et. al., 1992; Carr et. al., 1993). The selected dose of ß-FNA was chosen based on previous data showing pretreatment of mice with ß-FNA blocks [D-Ala^2, ME-Phe^4, gly(ol)^5]enkephalin- but not [D-Pen^2, D-Pen^5]enkephalin-induced analgesia in mice (Paul et. al., 1989). Another recent study has shown the δ-selective opioid receptor antagonist (E)-7-benzylidine-7-dihydronaltrexone (BNTX, Porteghese et. al., 1992) does not block morphine-mediated

suppression of SL or PL CTL activity (Carr & Carpenter, submitted) suggesting μ- but not δ-opioid receptor involvement.

Morphine-mediated suppression of CTL activity in alloimmunized mice may be due to immune dysfunction at the cellular level. Serine esterases, such as the BLT esterase, have been identified in CTLs (Pasternack & Eisen, 1985), are released after specific target cell binding (Pasternack et. al., 1986), and have been localized to the cytotoxic granules (Young et. al., 1986) implicating these enzymes as a mechanism of target cell lysis by CTLs. Other studies have indicated that serine esterases are not necessary for target cell lysis depending on the target (Trenn et. al., 1987; Ostergaard et. al., 1987). By inhibiting serine esterase release from CTL clones using cyclosporine A, another laboratory has hypothesized the existence of a cyclosporine-sensitive capacity to induce target cell lysis and a cyclosporine-insensitive mechanism of inducing lysis of target cells that does not require granule exocytosis (Lancki et. al., 1989). Recently, a third mechanism of target cell lysis by $CD4^+$ and $CD8^+$ T cells has been identified which predominately involves direct TNF-α-dependent lysis of TNF-α-sensitive targets (Smyth & Ortaldo, 1993). A recent study showed serine esterase release and total cell serine esterase content was reduced in SL taken from chronic morphine-treated mice compared to vehicle-treated controls (Carpenter et. al., 1994). In addition, no differences were found in the number of SL conjugating with targets suggesting the conjugating process of CTLs generated over the course of 11 days was similar between vehicle- and chronic morphine-treated animals (Carpenter et. al., 1994). The suppression of serine esterase

release and total serine esterase content of lymphocytes from chronic morphine-treated animals is consistent with a role for this enzyme in lysing target cells. Similar results have also been obtained using IL-2- and IL-12-stimulated human $CD8^+$ T cells (Mehrotra et. al., 1993). In support of this hypothesis, SL CTLs from vehicle- and chronic morphine-treated mice lyse the IL-4 targets through a Ca^{2+}-dependent process (Carr & Carptenter, submitted) implicating granzyme A (contains serine esterases) in the "lethal hit." (Berke, 1994).Consequently, one explanation for the reduction in cytolytic capacity of lymphocytes chronically exposed to morphine in vivo is a reduced capacity to produce granules which contain serine esterases and/or an inability to exocytosis esterase-containing granules.

In the present study, chronic morphine exposure was found to elevate the percentages of both $CD4^+$ and $CD8^+$ cells in the spleen. SL Ig^+ cell numbers were not affected. These results conflict with previous data showing a time-dependent increase in the $CD4^+$ cells and decrease in the $CD8^+$ cells in the spleen (Arora et. al., 1990). The results of another study revealed a decrease in both $CD4^+$ and $CD8^+$ cells (Kimes et. al., 1992). The discrepancies in the results may be due to the time course of morphine treatment. In the present study, morphine administration was continued for 11 days while the other studies employed morphine pellet implants and measured T cell subsets 72-120 h post implantation (Arora et. al., 1990; Kimes et. al., 1992). Another difference between these investigations involves the different strains of mice employed. It has previously been shown that there are strain differences in susceptibility to morphine effects on

immune responses (Bussiere et. al., 1992). Our results indicate that chronic morphine treatment increases the numbers of both $CD4^+$ and $CD8^+$ cells in the spleen. Additional studies using CD markers expressed on these cells are in progress. Other studies using chronic morphine-treated rhesus monkeys has shown an increase in the percentage of $CD4^+CD29^+$ PBMCs and a decrease the percentage of $CD4^+CD45RA^+$ PBMCs (Carr & France, 1993). Such a shift in the memory/helper $CD4^+$ population may alter cytokine production affecting the generation of CTLs.

The data indicating that chronic morphine exposure acts selectively on antigen-driven cytolytic function and not NK cytolytic activity may implicate morphine in the regulation of cytokine production. Since CTL maturation and maintenance of function require IL-2 and in certain instances IL-6 (Bass et. al., 1993), morphine regulation of the production of these cytokines could affect CTL generation and activity. In support of this hypothesis, it is known that morphine-mediated suppression of the primary antibody response is the result of a reduction in IL-6 synthesis; exogenous IL-6 attenuated the suppression in antibody production (Bussiere et. al., 1993). Another study revealed that morphine pellet implantation has no effect on PMA-induced IL-2 production (Saini & Sei, 1993); it may be that morphine selectively inhibits specific cytokines. Whereas morphine may reduce the production of some cytokines, recent data indicates that it augments TGF-ß production <u>in vitro</u> (Chao et. al., 1992); this cytokine is a negative regulator of many T cell responses (Ishizaka et. al., 1992).

In summary, the data indicate that prolonged exposure to morphine diminishes the capacity to sustain CTL activity to alloantigenic cells. Since morphine (and heroin) has previously been shown to promote the growth of HIV-1 in mitogen-stimulated PBMCs in vitro (Peterson et. al., 1990; Adler et. al., 1993) and both reduce immune responses, the molecular mechanisms of action of opioids in affecting immune homeostasis will be a primary focus of future research.

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Table I.

Splenic CTL effector cells are $CD8^{+a}$

Treatment	Unfractionated	$CD4^+$-enriched	$CD8^+$-enriched
VEHICLE	17.7 ± 1.9[b]	1.8 ± 1.4	74.0 ± 6.4
MORPHINE	8.0 ± 1.6	1.6 ± 1.5	15.2 ± 5.3

[a]SL from vehicle- and chronic morphine-treated mice (n = 3/group) were enriched for either $CD4^+$ or $CD8^+$ cells (see Material & Methods section). Prior to enrichment, SL were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. Cells from each treatment group were pooled and subsequently enriched for $CD4^+$ or $CD8^+$ cells which subsequently were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. Between 80-90 % of SL were lost as a result of the enrichment step which is consistent with the fact that SL contain 8-12% $CD8^+$ cells (Fig. 4A). This table is a summary of two independent experiments with similar outcomes.

[b]Numbers are in LU ± SEM, n = 3.

Table II

ß-FNA partially antagonizes morphine-mediated augmentation of the SL proliferative response to PWM*

Treatment	Counts per minute ± SEM
VEHICLE	14542 ± 588
MORPHINE	20869 ± 611**
ß-FNA	13715 ± 83
MORPHINE + ß-FNA	17622 ± 644**†

*SL obtained from mice (n = 8/group) treated as described in the legend of figure 5 were cultured in the presence of PWM for 48 h. Two hundred nCi of ^{3}HTdR was added to the wells and cells were incubated an additional 12 h. Cells were harvested and assayed for ^{3}HTdR incorporation.

**$F(3,21) = 63.1663$, $p < .01$ comparing SL from chronic morphine-treated mice to vehicle-treated controls as determined by ANOVA and Scheffe multiple comparison test.

†$p < .05$ comparing SL from mice co-administered ß-FNA + morphine to chronic morphine-treated animals as determined by ANOVA and Scheffe multiple comparison test.

Figure 1. ***ß-FNA reverses morphine-induced suppression in splenic lymphocytes CTL activity.***

C3H/HeN mice (n = 8/group) were administered ß-FNA (40.0 mg/kg, s.c.) or vehicle 18-24 h prior to receiving morphine (50.0 mg/kg, s.c.) followed by alloimmunization (1 X 10^7 C57BL/6J splenocytes, i.p.) 2 h later. Mice received morphine (50.0 mg/kg, s.c.) or vehicle daily for the next 10 days. In addition, mice received ß-FNA (40.0 mg/kg, s.c.) every 72 h. Mice were re-immunized (1 X 10^7 C57BL/6J splenocytes, i.p.) 6 days following the initial immunization. The animals were sacrificed on day 11 and their splenic lymphocytes (SL) assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. Bars represent SEM, n = 8. *F(3,31) = 1.9646, p = .05 comparing vehicle- to chronic morphine-treated group as determined by ANOVA and Tukey's multiple comparison test.

Figure 2. ***Long acting μ-antagonist ß-FNA blocks morphine-induced suppression of CTL activity of peritoneal lymphocytes.***

Mice were treated as described in the legend of figure 4. Peritoneal lymphocytes (PL) were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. PL CTL activity from chronically morphine-treated mice was significantly suppressed relative to other treatment groups. ß-FNA co-treatment completely reversed this effect. *F(3.18) = 3.9754, p < .05 comparing chronic morphine-treated animals to all other groups of mice as determined by ANOVA followed by Scheffé multiple comparison test. Bars represent SEM, n = 7.

Figure 3. ***ß-FNA alone or in combination with morphine had no effect on splenic NK activity.***

Mice were treated as described in the legend of figure 5. Splenic lymphocytes (SL) were collected and assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM, n = 8.

Figure 4. ***Cytometric analysis of CD4$^+$- and CD8$^+$-enriched splenic lymphocytes.***

Splenic lymphocytes obtained from mice treated as described in the legend of figure 1 were labelled using anti-CD4 antibody conjugated to FITC and anti-CD8 antibody conjugated to PE. (A) Splenocytes prior to enrichment. (B) Splenocytes following CD8$^+$ enrichment. (C) Splenocytes following CD4$^+$ enrichment.

Figure 5. ***ß-FNA antagonizes morphine-mediated increases in splenic CD3$^+$CD4$^+$ and CD8$^+$ subpopulations.***

Mice were treated as described in the figure legend of figure 5.

A) ß-FNA antagonized the increase in the percentage of CD3$^+$CD4$^+$ splenic lymphocyte following chronic morphine administration.

*$F(1,6) = 4.9923$, $p < .05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffé multiple comparison test. All other groups were not significant compared to vehicle-treated animals. Bars represent SEM, n = 7.

B) ß-FNA antagonized the increase in the percentage of $CD8^+$ splenic lymphocytes following chronic morphine administration.

*$F(3,15) = 7.4202$, $p < .05$ comparing chronic morphine-treated mice to all other groups as determined by ANOVA and Tukey's t-test). Bars represent SEM, $n = 6$.

C) Chronic morphine exposure had no effect on the percentage of Ig^+ splenic lymphocytes. Bars represent SEM, $n = 7$.

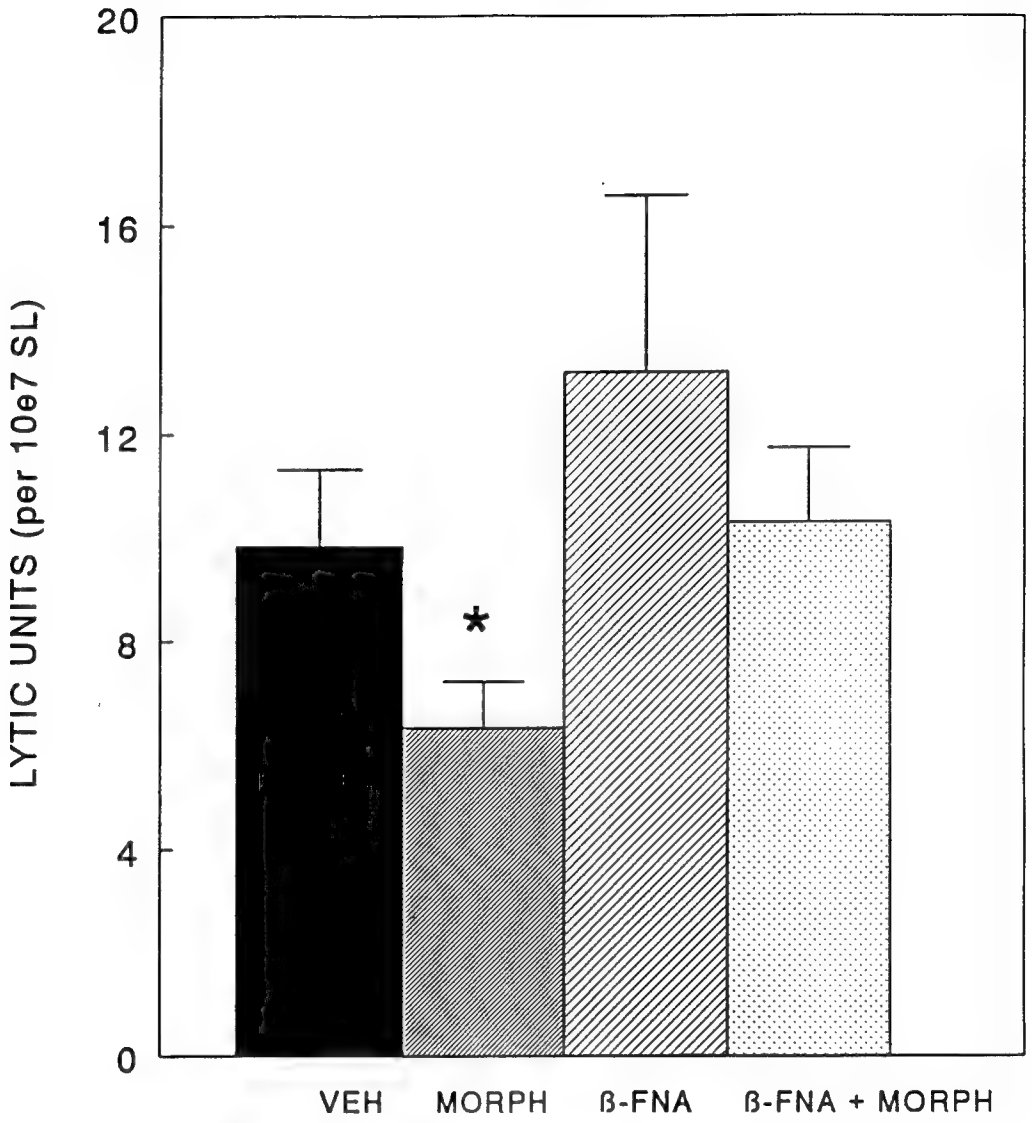


Fig 1

Carpl

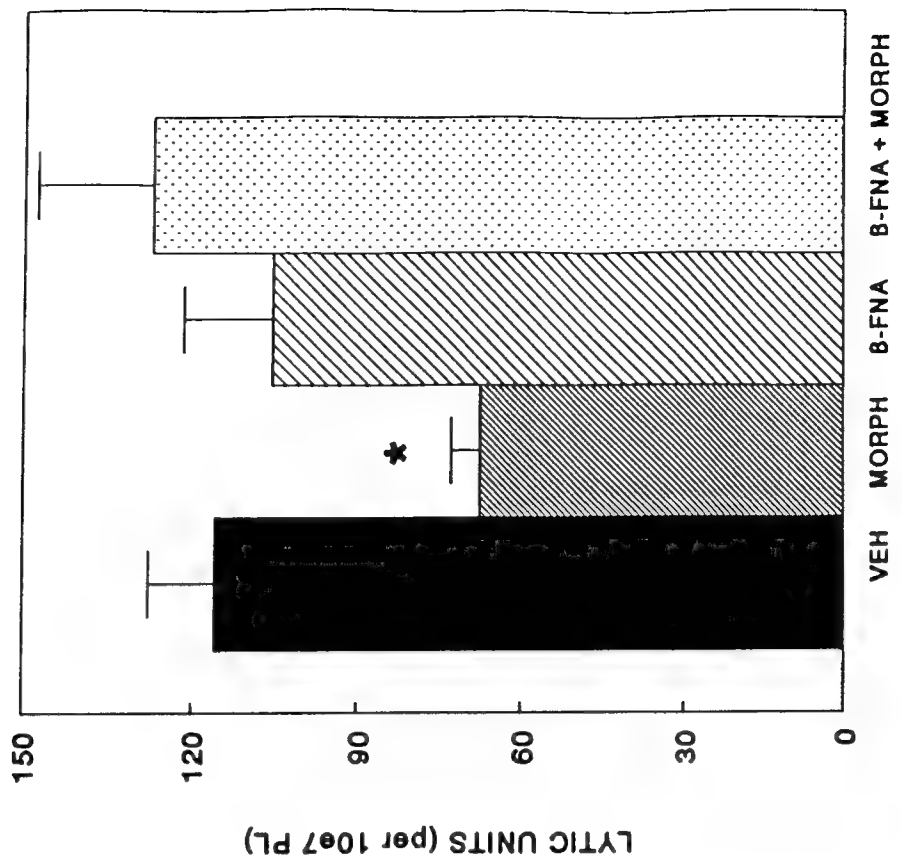


Fig 2

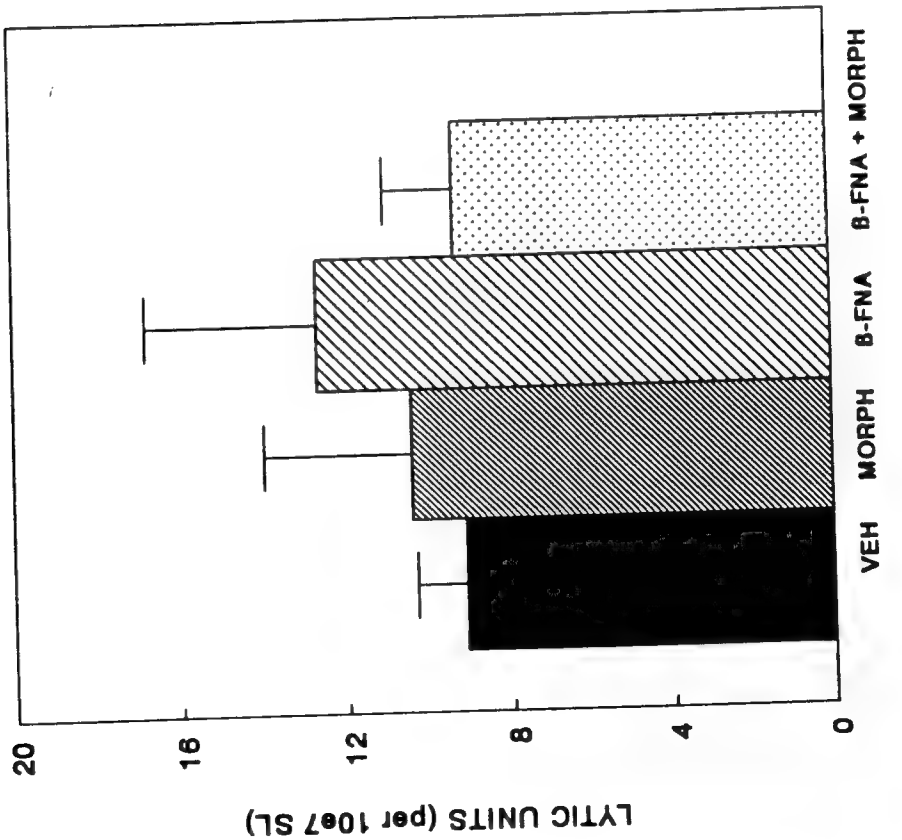


Fig 3

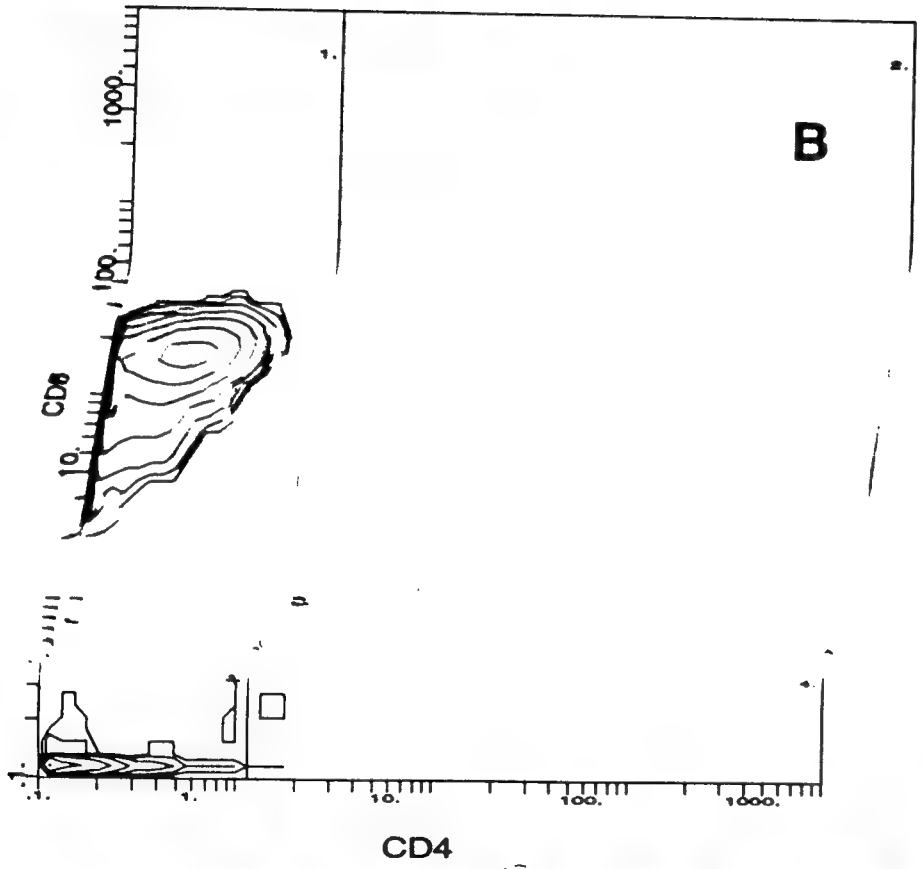
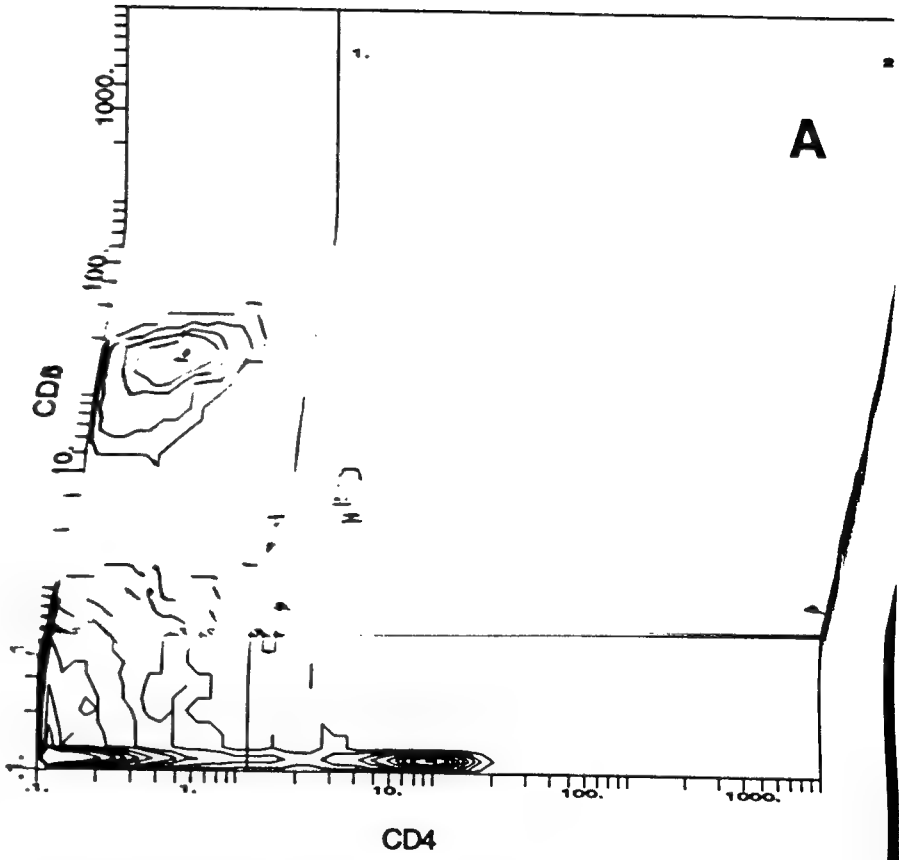

B
1000.
100.
10.
1.
CD8
1.
10.
100.
1000.
CD4

Fig 4B


A
1000.
100.
10.
1.
CD8
1.
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1000.
CD4

Fig 4A

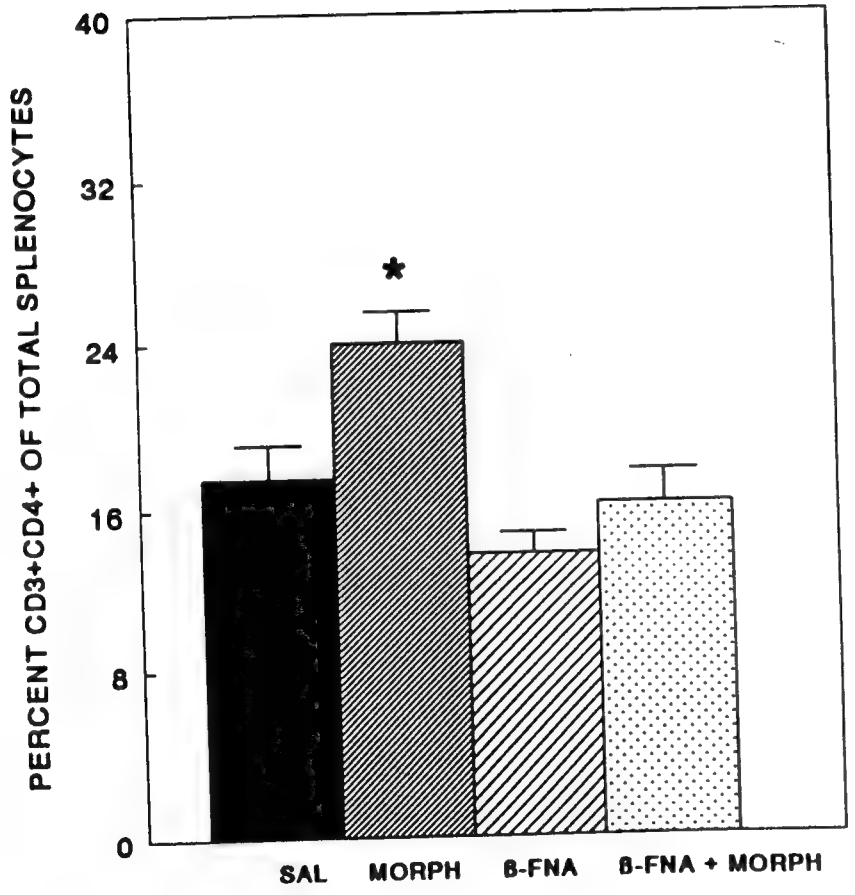
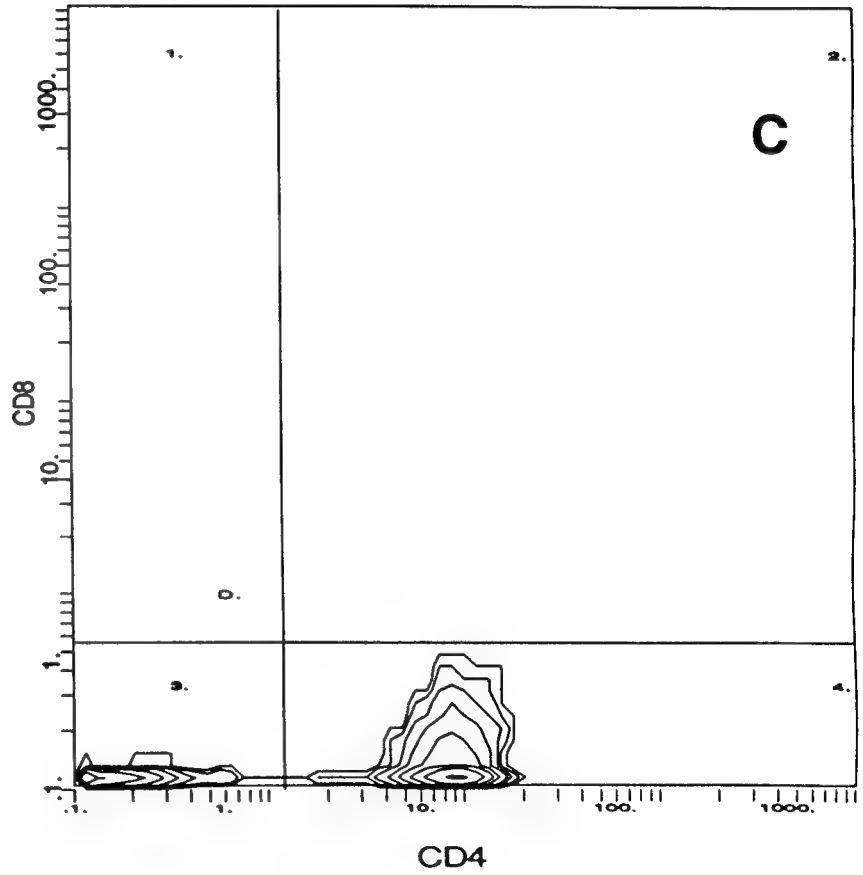

PERCENT CD3+CD4+ OF TOTAL SPLENOCYTES
40
32
24
16
8
0
*
SAL
MORPH
β-FNA
β-FNA + MORPH

Fig 5A


C
CD8
CD4
1000.
100.
10.
1.
0.1
0.1
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2.
3.
4.

Fig 4C

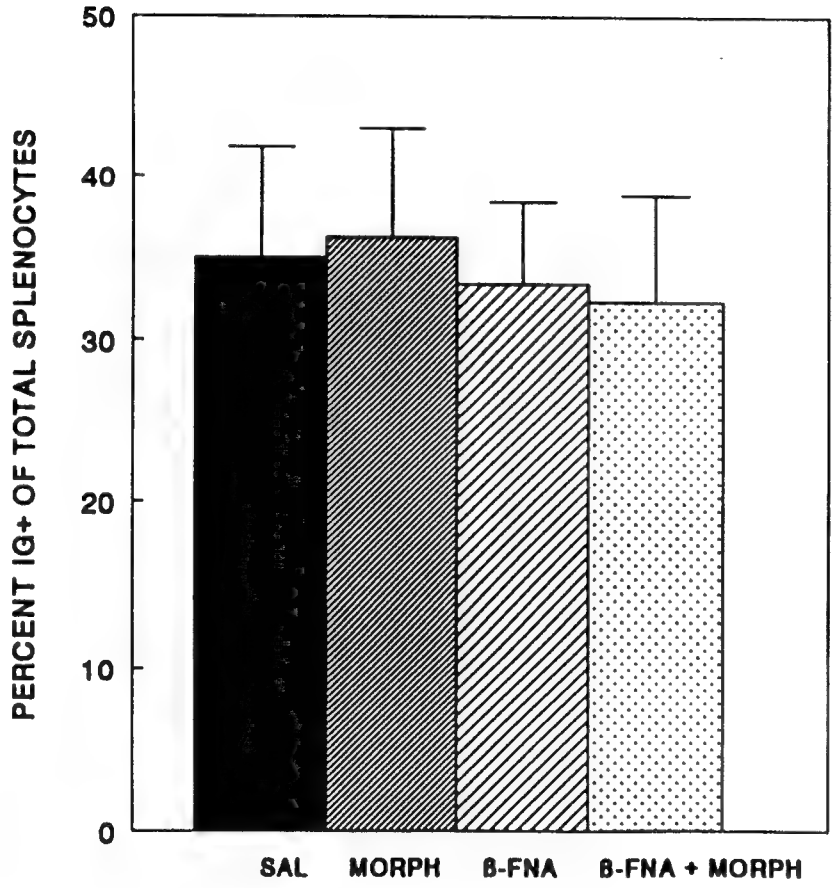
PERCENT IG+ OF TOTAL SPLENOCYTES
50
40
30
20
10
0
SAL
MORPH
B-FNA
B-FNA + MORPH

Fig 5c

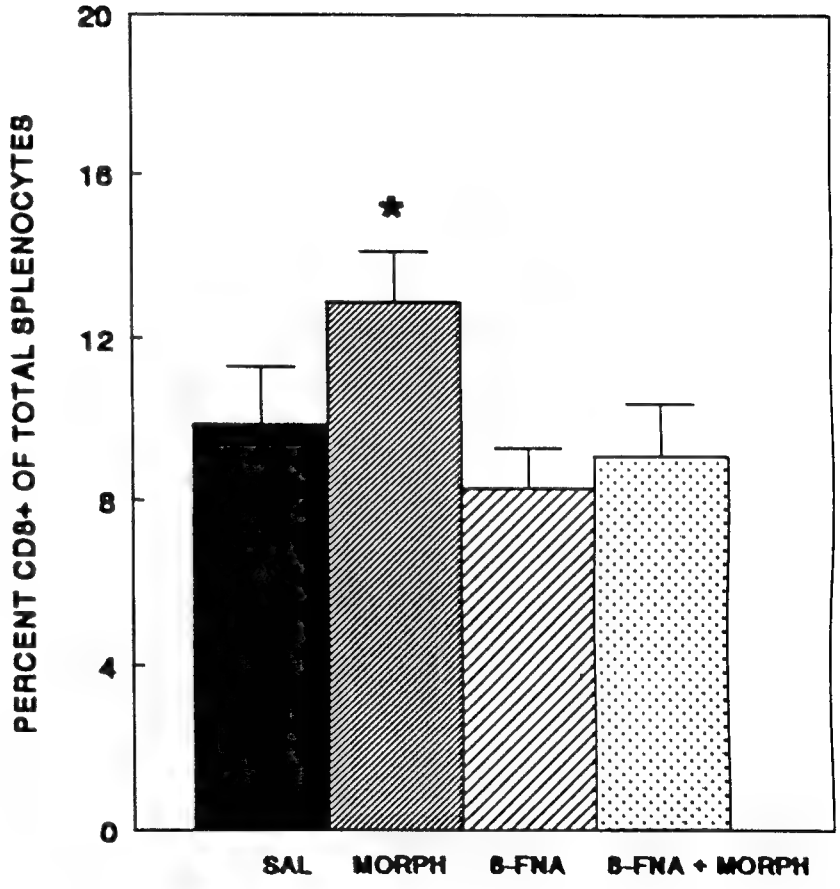
PERCENT CD8+ OF TOTAL SPLENOCYTES
20
16
12
8
4
0
*
SAL
MORPH
B-FNA
B-FNA + MORPH

Fig 5b

Submitted: Neuroimmunomodulation

Chapter 6
Appendix Item 7

Morphine-Induced Suppression of Splenic CTL Activity in Alloimmunized Mice is not Mediated Through a δ-Opioid Receptor

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Running head: Morphine-induced immunosuppression

key words: morphine, cytotoxic T lymphocyte, BNTX, opioid receptor, NK activity, neuroimmunomodulation

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ABSTRACT

The effect of chronic morphine exposure on natural killer (NK) activity *in vivo* and the generation of cytotoxic T lymphocytes (CTLs) *in vitro* and *in vivo* was investigated. Chronic exposure to morphine (10^{-5} -10^{-11} M) *in vitro* had no effect on the generation of antigen-driven effector cells. However, the daily administration of morphine (50.0 mg/kg, s.c.) into alloimmunized mice (C57BL/6 into C3H/HeN) for 11 days resulted in a decrease in peritoneal and splenic CTL activity but not splenic NK activity. In addition, there was a 60% decrease in the number of thymocytes recovered from chronic morphine-treated mice compared to vehicle-treated controls. However, the overall percentage of $CD4^+CD8^-$, $CD4^-$ $CD8^+$, and $CD4^+CD8^+$ thymocytes did not change between the two groups of treated animals. Pretreatment of the mice with the δ-selective antagonist, (E)-7-benzylidine-7-dihydronaltrexone (BNTX) did not block morphine-mediated suppression of splenic CTL activity but did block morhine-induced suppression of peritoneal lymphocyte CTL activity. In addition, BNTX pretreatment alone augmented splenic NK activity and such augmentation was blocked following chronic morphine exposure. Splenic CTL effector cells from either treated group of animals lysed their target (EL-4 lymphoma) through a Ca^{2+}-dependent mechanism. Collectively, the results indicate morphine suppresses splenic CTL activity through an indirect pathway, insensitive to δ-opioid receptor antagonists rather than through direct lymphocyte opioid receptors.

INTRODUCTION

The abuse of opioids (e.g., heroin and fentanyl) resulting in a compromised immune system [1-3] and a greater susceptibility to infectious agents [4,5] has made this class of compounds a liability and predicted co-factor in the acquisition and spread of HIV-1 [6]. The implicated co-factor relationship between opioids and HIV-1 is supported by data showing morphine amplification of HIV-1 expression in phytohemagglutinin-activated peripheral blood mononuclear cells [7]. In addition, morphine increases lipopolysaccharide (LPS)-primed microglial production of tumor necrosis factor (TNF)-α which in turn promotes HIV-1 expression in latently infected promonocytes [8] and promonocyte-fetal brain cell co-cultures [9]. Within the immune system, chronic morphine treatment has been shown to increase the percentage of $CD4^+CD29^+$ peripheral blood T lymphocytes in rhesus monkeys [10] which are the reported reservoirs for simian immunodeficiency virus [11]. At the molecular level, pokeweed mitogen-stimulated peripheral blood mononuclear cells from rhesus monkeys treated chronically with morphine possess elevated levels of NFκB which is a regulatory element for key cytokines including interleukin (IL)-2 and TNF-α and binds to sites within the HIV-1 promoter [Carr et. al., submitted]. Taken together, the data would suggest opioids increase transcriptional regulators which promote HIV-1 replication, augment the reservoir cell population for HIV-1 replication in the peripheral blood, and increase cytokine production by microglial cells in the brain increasing the likelihood of replication of the virus in the central

nervous system. However, the effector cell population (CTLs) which is typically responsible for monitoring virus infection has only recently been evaluated in the presence of opioids.

Previous studies have shown the endogenous opioid peptides ß-endorphin and [met]-enkephalin augment the generation of CTLs in one-way mixed lymphocyte cultures (MLCs) [12]. More recently, alloimmunized mice chronically treated with morphine (50.0 mg/kg s.c., daily for 11 days) presented with significantly suppresses CTL activity in splenic and peritoneal lymphocyte populations [13]. Moreover, the μ-selective opioid receptor antagonist, ß-funaltrexamine (ß-FNA) blocked the suppression of CTL activity in the chronic morphine-treated mice suggesting the effect was mediated in part by μ-opioid receptors [Carpenter & Carr, submitted]. The mechanism of this suppression was identified to include a decrease in the production and release of serine esterases which are typically associated with the "lethal hit" by a proportion but not all CTLs [13]. The release of granules (granzyme A) containing the serine esterases is a Ca^{2+}-dependent phenomena while the other mode of CTL-directed lysis of target cells involves receptor-mediated apoptosis which does not require extracellular Ca^{2+} [14].

To further the initial observations of morphine-mediated suppression of CTL activity, we investigated: (i) the specificity of opioid-induced suppression; (ii) the

predominate pathway of CTL-directed cytolysis of target cells; and (iii) T cell precursor development through the assessment of thymic subpopulations in chronic morphine-treated mice.

MATERIALS AND METHODS

Mice and tumor lines

Female C57BL/6 and C3H/HeN (Harlan Sprague Dawley, Indianapolis, IN) mice were housed in groups of 5 per cage and maintained on a 12 h light/dark cycle. Access to water and food (Purina Mouse Chow) was available *ad lib.* The YAC-1 and EL-4 mouse lymphoma cell lines and P815 mastocytoma cell line were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD); the cells have been maintained in culture by biweekly passage over a six month period. All animals used in these studies were maintained in accordance with the Committee on the Use and Care of Animals, Louisiana State University Medical Center and the guidelines of the Committee on Care and Use of Laboratory Animals Resources, National Research Council, Department of Health, Education, and Welfare Publications Number (National Institutes of Health) 85-23, revised 1985.

Morphine treatment regimen

A dose-response study has established that 50.0 mg/kg of morphine s.c. results in maximal suppression of cytolytic activity [15]. Consequently, this dose was used in the *in vivo* experiments.

C3H/HeN mice (n = 10/group) were administered vehicle or the δ-opioid receptor antagonist BNTX (0.6 mg/kg, s.c.) 30 minutes prior to the initiation of the chronic morphine treatment. Morphine was administered 2 h prior to receiving 1 X 10^7 C57BL/6 spleen cells, i.p. Following the immunization, mice received vehicle or morphine daily for an additional 6 days. On day 7, mice were re-immunized with 1 X 10^7 C57BL/6 spleen cells, i.p. 2 h after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional 3 days. In addition to the daily administration of morphine or vehicle, mice received either vehicle or BNTX (0.6 mg/kg, s.c.) daily 30 minutes prior to morphine administration. On day 11 after the initial immunization, the mice were sacrificed and PL and SL were assayed for CTL and NK activity.

Lymphocyte and thymocyte preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hank's balanced salt solution (HBSS). Cells were collected by recovering 10 ml of peritoneal fluid through a 20 gauge needle and 10 ml syringe. Thymus and spleens were removed and cell suspensions were prepared by mechanical dispersion. SL, PL and thymocytes were washed with HBSS (250 x g, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4CL; the cells were subsequently washed with HBSS (250 x g, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybri-max (Sigma Chemical

Co., St. Louis, MO) antibiotic/antimycotic solution (complete media). Cells were counted and examined for viability via trypan blue exclusion dye.

In vitro generation of cytotoxic effector cells

Sterile suspensions of SL were prepared, and red blood cells were osmotically lysed as described above. In vitro cultures were established in sterile flat-bottomed 24-well microtiter plates (Costar, Cambridge, MA) with complete media. C3H/HeN SL (6×10^6 cells) were co-cultured for 72-120 hours with irradiated (900 rads) stimulator (C57BL/6) cells (4×10^6 cells) in the presence or absence of morphine (10^{-5} - 10^{-11} M). The indicated amount of morphine was added daily to the cultures. These cultures were incubated in 5% CO_2 atmosphere at 37°C. At the end of the culture period, cells were pelleted (200 x g, 5 min), washed with HBSS, and resuspended in a volume of complete media to give the effector-to-target cell ratios of 40:1, 20:1, 10:1, and 5:1. Each effector-to-target cell ratio was determined in triplicate. The percent killing at each effector-to-target ratio was converted to lytic units (LU). One LU was defined as the number of effector cells able to lyse 30% of the targets (EL-4 lymphomas) and this unit was expressed per 10^7 cells.

^{51}Cr-release cytolytic assay

SL and PL CTL activity was assayed using a 4 h microcytotoxicity assay with ^{51}Cr-labeled EL-4 cells (H-2^b) or P815 cells (H-2^d) as targets. ^{51}Cr-labeled YAC-1

lymphoma cells were used as targets to measure SL NK activity. Between 2 x 10^4 and 100 X 10^4 effector cells were mixed with 1 X 10^4 target cells in conical 96-well microtiter plates (Costar, Cambridge, MA) in a reaction volume of 0.2 ml of complete media. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100 μl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percent cytolytic activity = [(experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release - spontaneous ^{51}Cr release)] X 100 where "spontaneous" refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr in the supernate of target cells lysed with 0.1% t-octylphenoxypolyethoxyethanol (Triton X-100) in complete medium. Each effector-to-target cell ratio was measured in triplicate/animal. The percent lysis was then converted to LU. One LU was defined as the number of SL or PL able to lyse 20% of the target cells and this unit was expressed per 10^7 cells. To determine antigen specificity for the in vitro generated CTL assay, P815 mastocytoma cells were ^{51}Cr-labeled and used as targets in the 4 h microcytotoxicity assay. To determine the Ca^{2+} requirement in the CTL-directed cytolysis of the target cell, SL CTLs were incubated in the presence or absence of 3.6 mM ethyleneglycol-bis-(ß-aminoethylether)N,N'-tetra-acetic acid (EGTA) during the 4-hour microcytotoxicity assay.

Fluorescence activated cell sorter (FACS) analysis of thymocyte populations

Thymocytes from the vehicle- or drug-treated groups of mice were collected and washed in RDF buffer (R & D Systems, Minneapolis, MN) and resuspended in 20 μl of RDF buffer containing fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (IgG_{2b} isotype, Gibco BRL, Gaithersburg, MD) and phycoerythrin (PE)-conjugated rat anti-mouse CD8 (IgG_{2b} isotype, Gibco BRL). FITC- and PE-conjugated rat IgG_{2b} were used as isotypic controls. The cells were allowed to incubate for 20-25 minutes on ice in the dark. Nine hundred microliters of RDF buffer was added to the cells which were then centrifuged (250 x g, 5 min). The supernatant fluid was discarded and the cell pellet was resuspended in 250 μl of RDF buffer and 250 μl of 2% paraformaldehyde (Sigma Chemical Company) and analyzed by FACS for the percentage of stained cells in the cell population. Light scatter was collected at 488 nm and the emitted light which passed through a long pass filter was analyzed at 525 nm (FITC) or 575 nm (PE) on a Coulter Elite FACS (Coulter, Hialeah, FL). Five thousand gated events were collected and analyzed per sample. Compensation between FITC and PE amounted to 20-25%.

Reagents

Morphine sulfate was generously provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). The δ_1-selective opioid receptor antagonist BNTX was purchased from Research Biochemicals Inc. (RBI,

Natick, MA). The drugs were dissolved in 10% dimethyl sulfoxide in HBSS. Vehicle consisted of 10% DMSO in HBSS.

Statistics

One-way ANOVA (Randomized, block design) was used together with Tukey's protected T test or Scheffe multiple comparison tests in comparing individual means between treated groups of animals in order to determine significance ($p < .05$). This statistical package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

Morphine Exposure In vitro Has No Effect on the Generation of Antigen-Driven Effector Cells

One-way MLCs were set-up to determine the direct effects of morphine on the generation of cytolytic effector cells. The results show morphine (10^{-5} - 10^{-11} M) added to cultures daily had no effect on the production of cytolytic effector cells compared to vehicle-treated controls determined 72-, 96- and 120-h following initiation of culture (fig. 1). Cytolytic effector cells collected on day 5 following culture were also tested for target specificity using ^{51}Cr-labeled P815 mastocytoma cells (H-2^d haplotype). No measurable cytolysis of this cell line was detected (data not shown).

Chronic Morphine Treatment Antagonizes the BNTX-Mediated Augmentation of Splenic NK Activity

C3H/HeN mice chronically administered morphine exhibited lower SL and PL CTL activity compared to vehicle-treated controls (fig. 2 & 3 respectively). Consistent with previous results, chronic morphine exposure had no effect on splenic NK activity (fig. 4). Pretreatment with BNTX antagonized morphine-induced suppression of PL CTL activity (fig. 3) but not SL CTL activity (fig. 2). Pretreatment of mice with BNTX alone had no effect on SL (fig. 2) or PL (fig. 3) CTL activity. However, BNTX treatment alone resulted in a significant increase in

splenic NK activity (fig. 4). In animals chronically treated with both morphine and BNTX, no measurable increase in splenic NK activity was observed (fig. 4).

Chronic Morphine Treatment Results in Thymic Atrophy but not a Disproportionate Increase or Decrease in the Percentage of Thymocyte Subpopulations

Since our results indicated morphine exposure suppresses a central T cell function, we investigated the progenitor T cells found in the thymus. Chronic morphine treatment significantly reduced the absolute number of cells recovered from the thymus. Specifically, there was a 60% decrease in recovered cells from chronic morphine-treated mice ($5.5 \pm 0.6 \times 10^7$ thymocytes) compared to vehicle-treated controls ($13.38 \pm 0.98 \times 10^7$ cells) ($p < .01$, ANOVA and Scheffe). However, the percentage of $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^+CD8^+$ cells within the thymus did not change (fig. 5).

CTLs Generated in the Alloimmunized C3H/HeN mice Lyse Their Targets Through a Ca^{2+}-Dependent Mechanism

There are currently two proposed mechanisms of lymphocyte-driven cytotoxicity: (i) non-secretory and (ii) secretory [14]. CTLs operate through either the Ca^{2+}-dependent membranolytic pathway [16] or a receptor-driven apoptotic-inducing mechanism which does not involve extracellular Ca^{2+} [14]. To identify which mechanism of CTL-directed cytolysis of target cells was utilized by the effector cells, SL were assayed for cytolysis of EL-4 target cells in the presence or absence

of EGTA. The results show SL from morphine- and vehicle-treated animals lyse their targets through a Ca^{2+}-dependent process (fig. 6).

DISCUSSION

Consistent with previous findings, the present study shows chronic treatment of mice with morphine resulted in a lower immune response to alloimmunization as reflected by CTL activity in both PL and SL populations. Pretreatment of mice with the δ-selective opioid receptor antagonist BNTX [17] did not block morphine-induced suppression of SL CTL activity but did antagonize morphine-mediated suppression of PL CTL activity. Together with the previous data showing ß-FNA pretreatment blocks morphine-mediated suppression of SL and PL CTL activity [Carpenter & Carr, submitted], the data suggest morphine-mediated suppression of SL but not PL CTL activity is mediated through a μ-opioid receptor. However, the results showing BNTX antagonizes morphine-mediated suppression of PL CTL activity similar to ß-FNA seems to suggest both δ and μ opioid receptors are involved either centrally (brain) or peripherally (spinal). The relationship between δ- and μ-selective antagonists and immunocompetence is currently unknown. The data indicating that morphine blocks the augmentation in splenic NK activity following chronic BNTX administration seems to suggest μ and δ opioid receptors may share a common pathway distal to the opioid binding site. The level of this interaction may be related to the level of action of these compounds. BNTX has been shown to act spinally through δ_1-type opioid receptors [18] which may also include μ_1 opioid receptors while morphine binds preferentially to μ_2 opioid receptors [19]. Although μ and δ opioid ligands have different sites of action

within the central nervous system relative to inducing or antagonizing analgesia, the relationship of the opioid receptor types on immunocompetence in vivo has not been determined. Recently, two studies suggest opioid-mediated analgesia and immunomodulation are functionally independent. Specifically, the administration of morphine into the anterior hypothalamus has been shown to inhibit blood lymphocyte proliferation but have no measurable analgesic action [20]. In a second study, mice pretreated with naltrexone (10.0 mg/kg, s.c.) and subsequently administered increasing increments of morphine up to 100.0 mg/kg showed appreciable analgesia (50-60% of maximal effect) but no suppression of splenic NK activity [15]. Taken together, it is tempting to speculate the existence of opioid receptors which can be distinguished by analgesic versus immunomodulatory activities. This being the case, it may be possible to identify an opioid compound which induces analgesia without the immunosuppressive side-effects. Recently, one such compound OHM3295, a fentanyl derivative, has been found to induce analgesia without suppressing splenic NK activity through a naltrexone-sensitive pathway [21]. In fact, OHM3295 augmented splenic NK activity in a dose-dependent fashion. Consequently, future work is necessary to determine the action of central (supraspinal) and peripheral (spinal) opioid receptors relative to analgesia and immunocompetence.

The concentration of BNTX used in this study was found to be fully antagonistic to [(D-Pen^2, D-Pen^5)enkephalin] (δ-opioid receptor selective agonist) in mice [18]. The

present investigation shows mice administered BNTX daily over 11 days had elevated levels of splenic NK activity. This increase could be due to a redistribution of NK cells from the circulation into the spleen or the activation of pre-NK cells to fully competent cytolytic cells. The observation that mice chronically treated with an opioid antagonist have elevated levels of splenic NK activity is not a novel finding. A previous study has shown mice chronically treated (168 h) with naloxone (0.1 - 1.0 mg/kg, s.c.) displayed increased levels of splenic NK activity compared to vehicle- or (+)-naloxone-treated control mice [22]. Taken together, the results imply endogenous opioid pathways are important in the regulation of immune homeostasis.

In the present study, SL CTL effector cells incubated with EGTA during the ^{51}Cr-release microcytotoxicity assay did not exhibit cytolytic activity suggesting a requirement for Ca^{2+}. These results complement previous work showing SL from morphine-treated mice possess significantly lower levels of serine esterases [13] which are utilized in the Ca^{2+}-dependent, secretory CTL-directed lysis of target cells [14]. Therefore, the membranolytic pathway used by effector cells generated in the alloimmunized C3H/HeN mice is altered following chronic morphine treatment. Currently, it is not known at what level the modification in the membranolytic pathway is affected by morphine. However, an aberrant response in the generation of cAMP following exposure to alloantigen by $CD8^+$-enriched effector cells taken from morphine-treated animals has been reported [13].

Morphine-mediated suppression of CTL activity is not simply due to a direct interaction of drug with lymphocytes. Although lymphocytes have been shown to possess opioid receptors [23] and recently, oligonucleotide primers specific for the δ-class opioid receptor cloned from the NG108-15 neuroblastoma X glioma cell line [24] has been used to generate a 400-bp product that has 100% homology with a published opioid receptor clone from brain [Halford, Gebhardt, Carr, unpublished observation], morphine was found to have no effect on the generation of CTLs in one-way MLCs. Therefore, similar to morphine-mediated suppression of splenic NK activity, morphine-induced suppression of CTL activity is indirect, potentially involving the hypothalamic-pituitary adrenal axis [25] and/or the sympathetic nervous system [26, 27].

The occurrence of thymic atrophy following morphine exposure has previously been described by numerous laboratories [25, 28, & 29]. In one investigation, the administration of morphine resulted in a time-dependent decrease in the $CD4^+CD8^+$ thymocyte population which recovered to normal levels by day 10 [29]. Consistent with these findings, our results show no change in the percentage of total double-positive thymocytes following the sacrifice of mice on day 11. The relationship between the initial decrease in thymocyte population following morphine exposure and the generation of CTLs in the spleen and peritoneum is currently unknown. However, observations showing elevated levels of $CD4^+$ and $CD8^+$ SL in the chronic morphine-treated mice [13] suggest an over

compensation in the peripheral T cell population. This over compensation might, in part, be due to the inability of the immune system from the morphine-treated mice to clear the antigen. The clearance defect could be the result of dysfunctional cytolytic activity mediated by the CTL effector cells as reported in the present study, inappropriate processing and presentation of antigen to pre-CTLs by macrophages, or a combination of both processes. Previous studies indicate a decrease in the index of phagocytosis by peritoneal and splenic macrophages following morphine exposure substantiating the notion of a clearance defect [30-32].

In summary, the present study indicates the continuous exposure to morphine has detrimental consequences on CTL activity implying opioid abusers are at a greater risk for the acquisition of viral infections. Controlled, experimental studies have shown mice exposed to morphine succumb to viral infections earlier and in greater numbers than vehicle-treated controls [13, 33]. These findings are consistent with published results in the human population [4, 5] supporting the supposition of opioids as co-factors for viral infections including AIDS [6]. Future work is required to identify the intracellular signalling pathways of effector cells modified by opioids following in vivo exposure as well as the neuroendocrine signals elicited by morphine either centrally or peripherally which ultimately affect the immune system. Pending the outcome of these findings, pharmacological intervention seems possible either through the development of novel analgesics which do not

activate those neuroendocrine pathways involved in immunomodulation or chemical antagonists which block the opioid-induced immunosuppression but not the analgesia.

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Figure 1. Chronic morphine exposure has no direct effect on the generation of antigne-driven effector cells in one-way mixed lymphocyte cultures.

C3H/HeN SL (6 X 10^6 cells) were co-cultured with irradiated (900 rads) C57BL/6 SL (4 X 10^6 cells) in the presence or absence of the indicated concentration of morphine added daily to each culture. Vehicle was added to control cultures. Following 72 hours (A), 96 hours (B), or 120 hours (C) culture incubation periods, the cells were harvested, enumerated and assayed for cytolytic activity using ^{51}Cr-labeled EL-4 cells as targets. Bars represent standard error of the mean, n = 5.

Figure 2. BNTX does not antagonize morphine-mediated suppression of splenic CTL activity.

C3H/HeN mice (n = 9/group) were administered BNTX (0.6 mg/kg, s.c.) or vehicle 30 min prior to receiving morphine (50.0 mg/kg, s.c.) or vehicle. Two hours after morphine administration, mice were alloimmunized (1 X 10^7 C57BL/6 splenocytes, i.p.). Groups of mice received morphine (50.0 mg/kg, s.c.) BNTX (0.6 mg/kg, s.c.), both morphine and BNTX, or vehicle daily for 10 additional days. All mice were re-immunized (1 X 10^7 C57BL/6 splenocytes, i.p.) 6 days after the initial immunization. Animals were sacrificed on day 11 and splenic lymphocytes (SL) were collected and assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. Chronic morphine or BNTX + morphine exposure significantly suppressed splenic CTL activity, $^{**}p < .01$ comparing drug-treated to vehicle controls as determined by ANOVA and Tukey's post T-test. Bars represent SEM.

Figure 3. BNTX antagonizes morphine-mediated suppression of peritoneal CTL activity.

Mice (n = 9/group) were treated as described in the legend of figure 2. Peritoneal lymphocytes (PL) were assayed for CTL activity using ^{51}Cr-labeled EL-4 cells. $^{\wedge}p = .06$ comparing morphine-treated to vehicle-treated controls. Bars represent SEM.

Figure 4. Morphine antagonizes BNTX-mediated augmentation of splenic NK activity.

Mice (n = 10/gorup) were treated as described in the legend of figure 2. Splenic lymphocytes (SL) were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Chronic BNTX exposure to mice resulted in potentiation of splenic NK activity. When mice were co-administered morphine and BNTX, splenic NK activity maintained levels similar to vehicle- or chronic morphine-treated animals. $^{*}p < .05$ comparing morphine-treated to vehicle-treated group as determined by ANOVA and Tukey's post T-test. Bars represent SEM.

Figure 5. Chronic morphine exposure does not modify the percentage of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, or $CD4^{+}CD8^{+}$ thymic subpopulations.

Mice (n = 6/group) were treated as described in the legend of figure 2. Upon sacrifice of the animals, thymocytes were collected, labeled as described in the Material & Methods section, and analyzed by FACS. Bars represent SEM.

Figure 6. SL CTL activity is Ca^{2+}-dependent.

Splenic lymphocytes (SL) obtained from either vehicle- or chronic morphine-treated mice (n = 3/group) were assessed for a Ca^{2+} requirement during the "lethal hit" by adding 3.6 mM EGTA to the media during the 4-hour microcytotoxicity assay. Bars represent SEM.

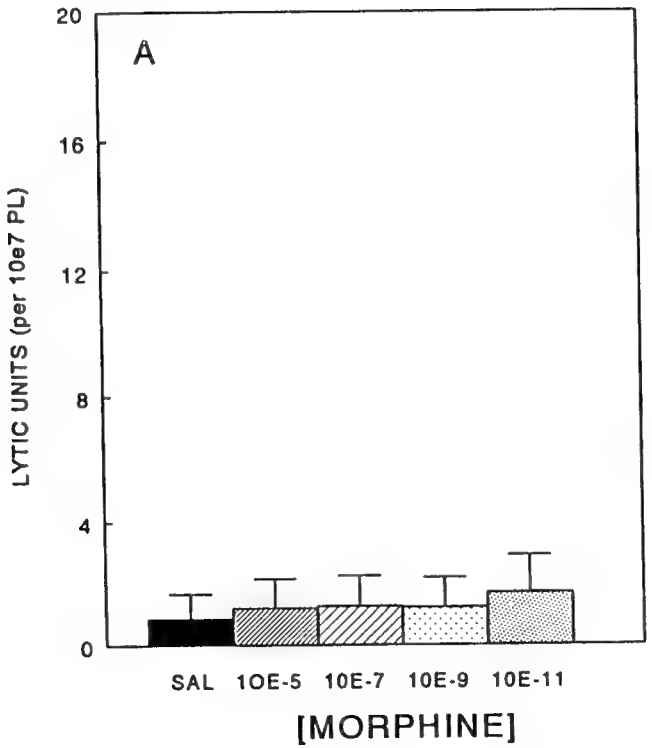

A
20
16
12
8
4
0
LYTIC UNITS (per 10e7 PL)
SAL
1OE-5
10E-7
10E-9
10E-11
[MORPHINE]

Fig 1 A

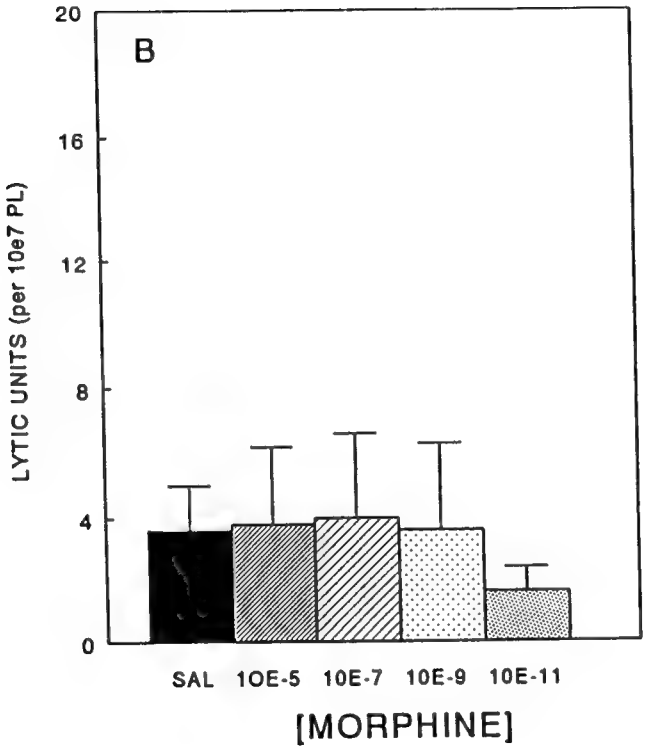
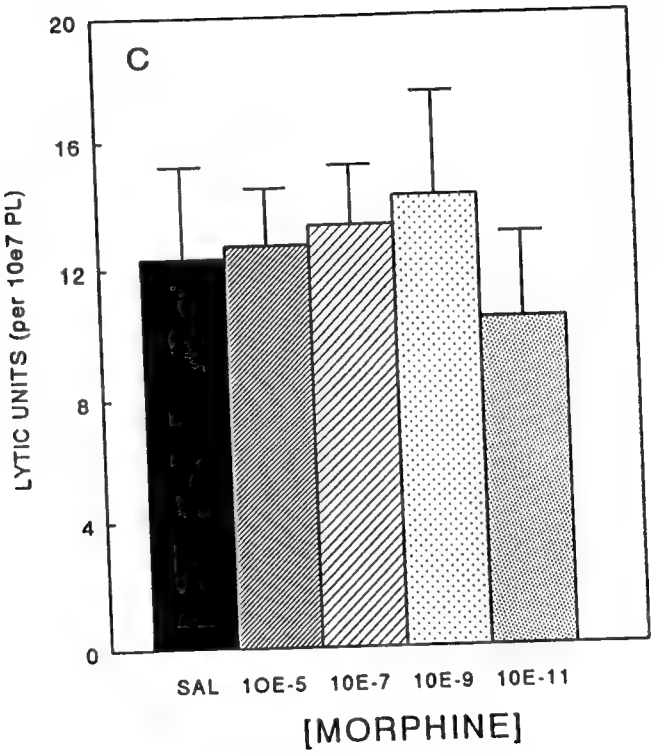

B
20
16
12
8
4
0
LYTIC UNITS (per 10e7 PL)
SAL
10E-5
10E-7
10E-9
10E-11
[MORPHINE]

Fig 1B


C
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16
12
8
4
0
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1OE-5
10E-7
10E-9
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[MORPHINE]

Fig 1c

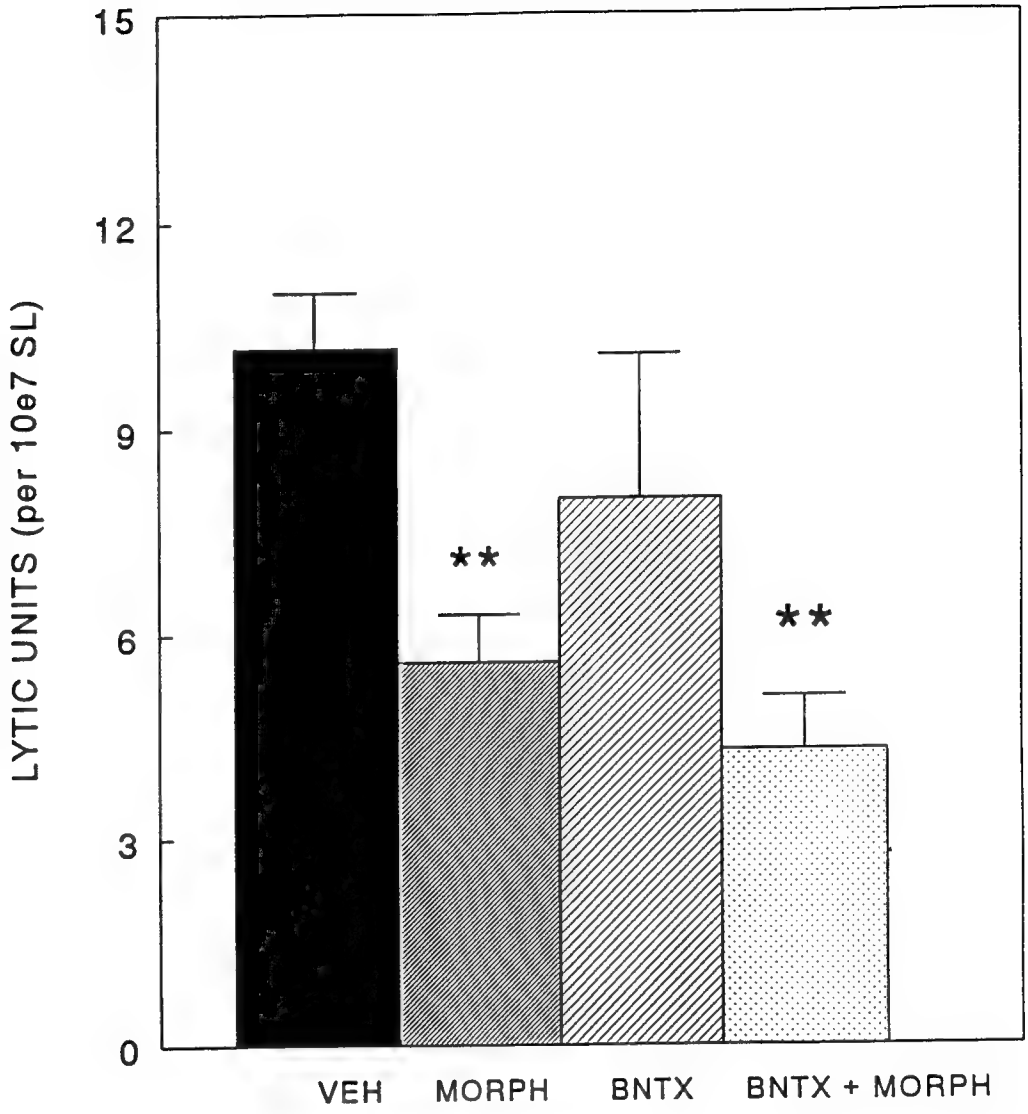
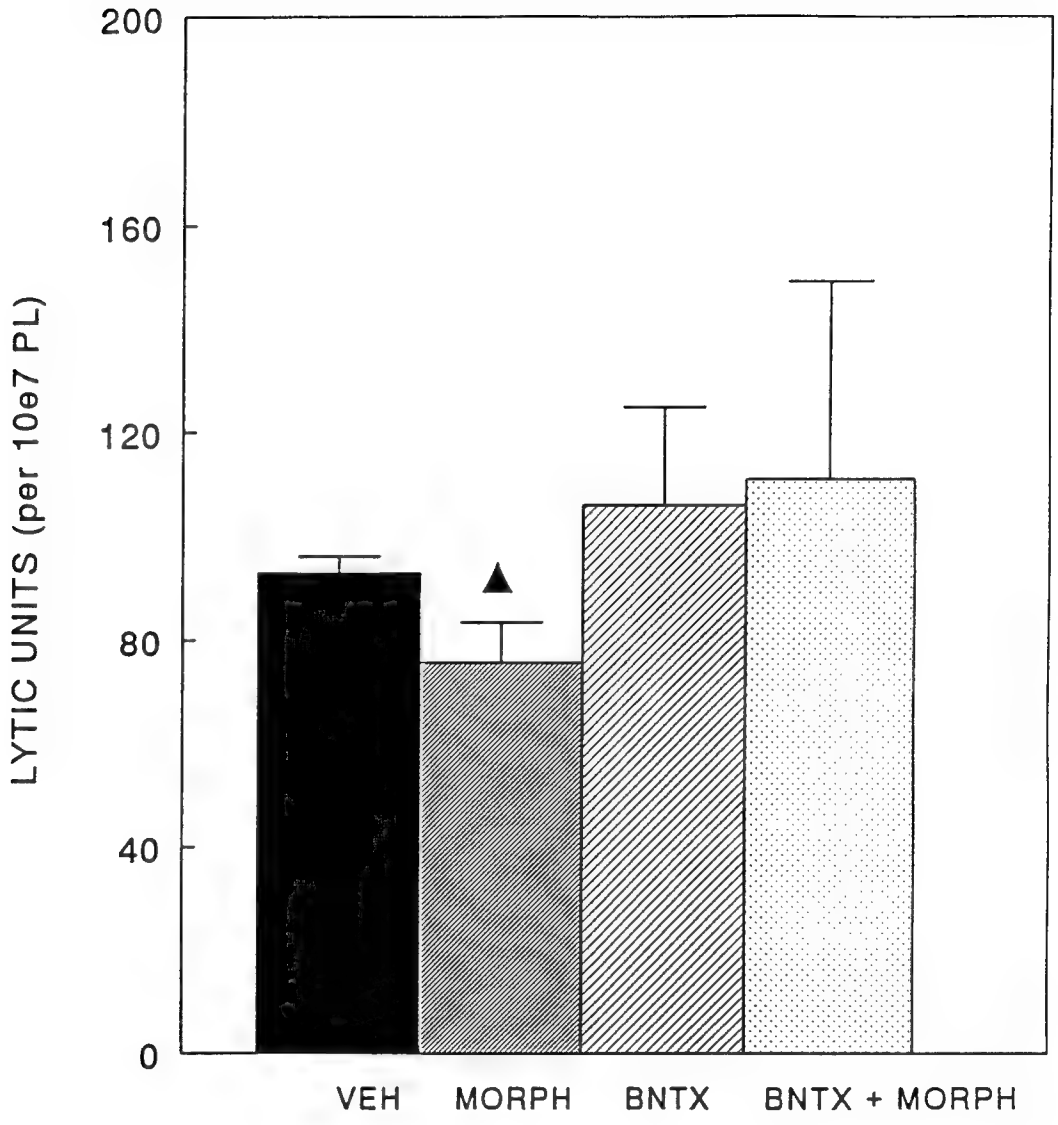


Fig 2


200
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120
80
40
0
LYTIC UNITS (per 10e7 PL)
VEH
MORPH
BNTX
BNTX + MORPH

Fig 3

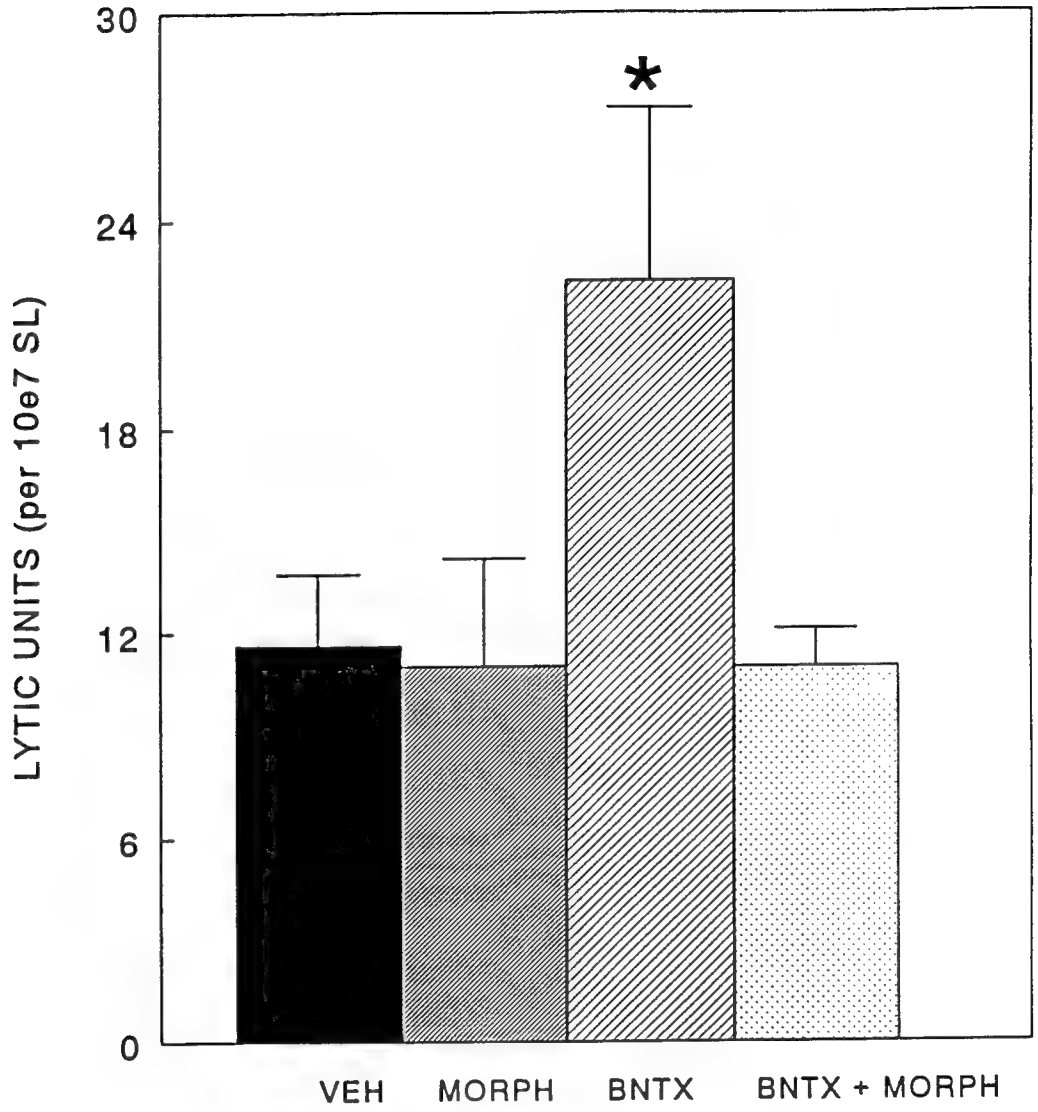
30
24
18
12
6
0
LYTIC UNITS (per 10e7 SL)
*
VEH
MORPH
BNTX
BNTX + MORPH

Fig 4

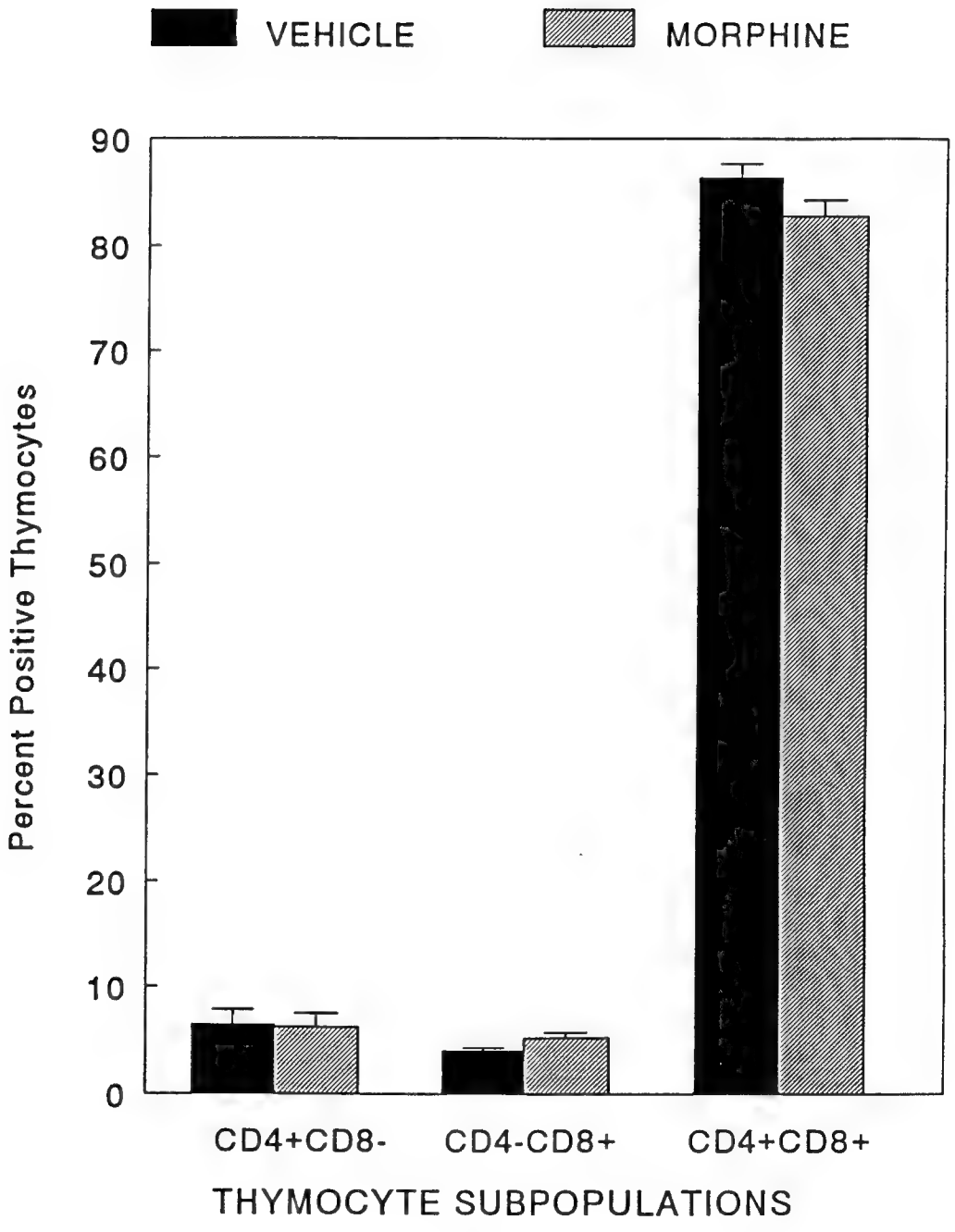
VEHICLE
MORPHINE
Percent Positive Thymocytes
90
80
70
60
50
40
30
20
10
0
CD4+CD8-
CD4-CD8+
CD4+CD8+
THYMOCYTE SUBPOPULATIONS

Fig 5

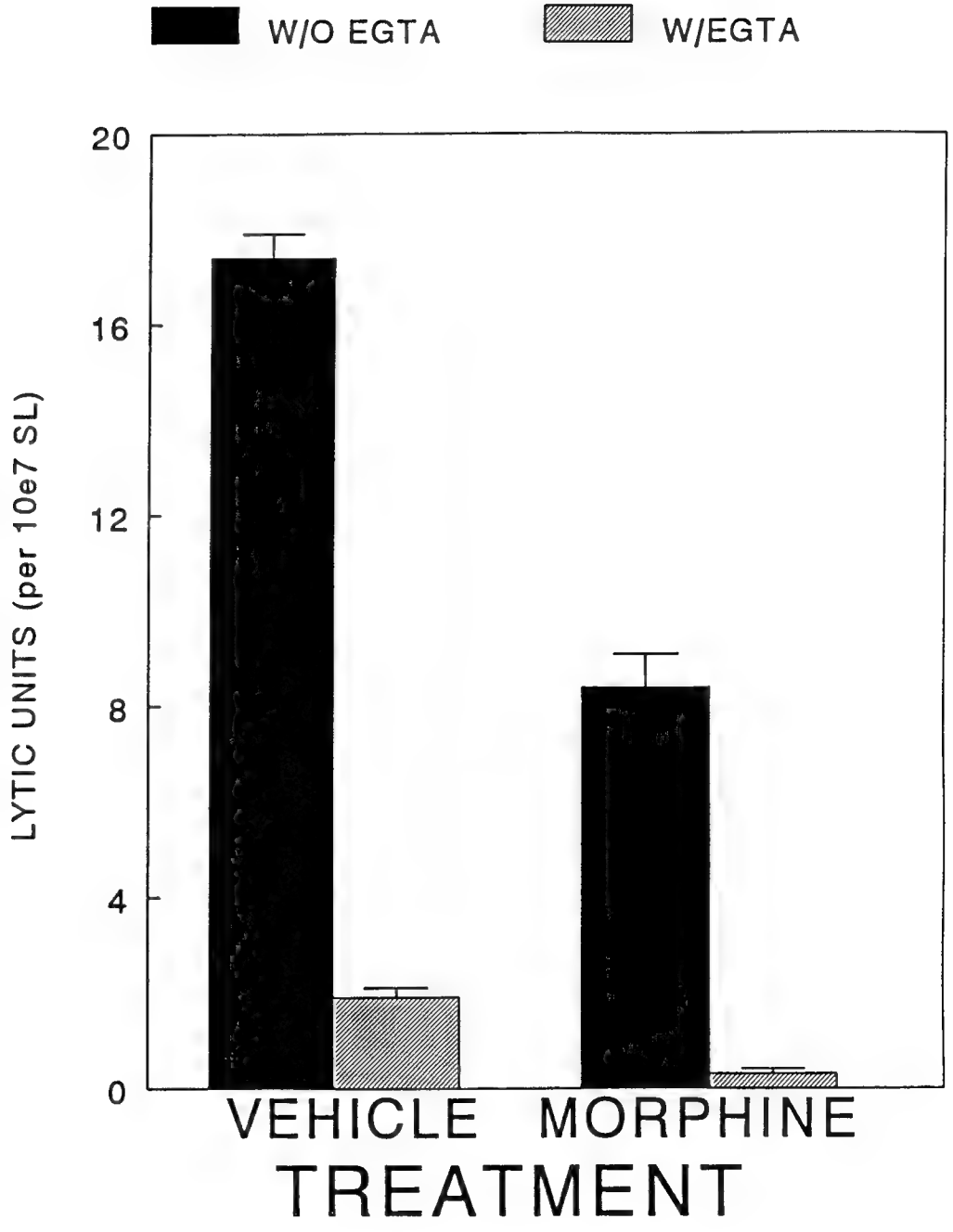
W/O EGTA
W/EGTA
20
16
12
8
4
0
LYTIC UNITS (per 10e7 SL)
VEHICLE
MORPHINE
TREATMENT

Fig 6

IN PRESS: BRAIN BEHAV. IMMUN.

Chapter 6
Appendix Item 8

Chronic Morphine Treatment Suppresses CTL-Mediated Cytolysis, Granulation and cAMP Responses to Alloantigen[1].

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ABSTRACT

Exposure to opioid drugs (e.g. morphine) *in vivo* has been shown to suppress natural killer (NK) cell activity. However, the effects of *in vivo* exposure to opioids on cytotoxic T lymphocyte (CTL) activity has not been investigated. The administration of morphine (50.0 mg/kg, s.c.) to alloimmunized mice for 11 days resulted in a significant decrease in peritoneal and splenic CTL activity. Moreover, the intracellular content of serine esterases and esterase release by $CD8^+$ effector cells from chronic morphine-treated mice was reduced compared to that of effector cells from vehicle-treated controls. In addition, the $CD8^+$ cAMP response to alloantigen was diminished compared to $CD8^+$ enriched cells from vehicle-treated animals. However, conjugate formation between effector and target and subsequent killing of target by effector cells did not reveal significant differences between vehicle- and chronic morphine-treated animals. Serum corticosterone and dehydroepiandrosterone levels were significantly lower in the chronic morphine-treated animals while proopiomelanocortin (POMC) gene expression (exon 3) in splenic lymphocytes did not correlate with morphine-mediated suppression of CTL activity. These results indicate that CTL activity is sensitive to chronic morphine exposure implicating opioids as important co-factors during viral infections in suppress cell-mediated immunity.

key words: Cytotoxic T lymphocytes, NK activity, morphine, serine esterase, cAMP, proopiomelanocortin, corticosterone.

INTRODUCTION

Short term (<120 hrs.) morphine administration has been shown to reduce NK activity (Shavit, Lewis, Terman, Gale, & Liebeskind, 1984; Bayer, Daussin, Hernandez, & Irvin, 1990), impair immunoglobulin production (Bussiere, Adler, Rogers, & Eisenstein, 1992; Pruett, Han, & Fuchs, 1992), suppress phagocytic activity (Levier, Brown, McCay, Fuchs, Harris, & Munson, 1993; Szabo, Rojivin, Bussiere, Eisenstein, Alder, & Rogers, 1993) and induce thymic hypoplasia (Fuchs & Pruett, 1993). In monkeys and humans, chronic morphine use is known to suppress NK activity (Novick, Ochshorn, Ghali, Croxson, Mercer, Chiorazzi, & Kreek, 1989; Carr & France, 1993).

The action of opioids on the nervous system has been proposed as the route to immunomodulation. Studies have shown that morphine can act centrally (Shavit, Depaulis, Martin, Terman, Pechnick, Zane, Gale, & Liebeskind, 1986) through receptors located in the periaqueductal gray matter of the mesencephalon (Weber & Pert, 1989). Two central nervous system (CNS) pathways have been shown to be involved in opiate-induced immunomodulation, the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system (SNS). The HPA axis has been implicated in short term studies of opioid-induced immunosuppression through the release of corticosterone (Bryant et. al, 1991; Sei, Yoshimoto, McIntyre, Skolnick, & Arora, 1991; Pruett et. al., 1992; Fuchs et. al., 1993; Migliorati, Nicoletti, D'Adamio, Spreca,

Pagliacci, & Riccardi, 1994). Endogenous corticosterone is selectively immunosuppressive suppressing interleukin (IL)-1, IL-2, and interferon (IFN) gamma production but acting synergistically with IFN gamma in enhancing monocyte activation antigen and the generation of reactive oxygen intermediates (for review Munck & Guyre, 1991). Corticosterone has also been implicated as the primary mediator of opioid-induced apoptosis of thymocytes resulting in thymic hypoplasia, a process reversible by adrenalectomy and mimicked by dexamethasone (Sei et. al., 1991). Furthermore, dexamethasone has been shown to induce apoptosis in mature NK and CTL cells *in vitro* and the addition of IL-2 or IL-4 protects against apoptosis (Migliorati et al., 1994).

The SNS is implicated in some forms of morphine-mediated immunosuppression through adrenergic pathways. Specifically, ß-adrenoceptor antagonists have been shown to block morphine-mediated suppression of mitogen-induced lymphocyte proliferation (Fecho, Dykstra, & Lysle, 1993). Alpha-adrenergic antagonists (and to a lesser extent, ß-adrenoceptor antagonists) have been shown to block suppression of splenic NK activity following acute morphine administration (Carr, Gebhardt, & Paul, 1993). Furthermore, recent data suggests central rather than peripheral adrenergic pathways are involved in morphine-mediated suppression of splenic NK activity (Carr, Mayo, Gebhardt, & Porter, 1994a). These observations coincide with previous data showing the

intracisternal administration of morphine resulted in the elevation of serum norepinephrine (NE), epinephrine (EPI) and dopamine from SNS stimulation of the adrenal medulla. The increase in monoamines was blocked by naloxone and the selective de-innervation of the adrenals (Van Loon, Appel, & Ho, 1981; Appel, Kirtsy-Roy, & Van Loon, 1986). Autonomic innervation of primary and secondary lymphoid tissue (Felten, Felten, Carlson, Olschowka, & Livnat, 1985) and the presence of α and ß adrenergic receptors on lymphocytes have been demonstrated (McPherson, & Summers, 1982; Fuchs, Albright, & Albright, 1988) suggesting direct effects of catecholamines on cells of the immune system. Moreover, a decrease in the affinity and increase in the number of ß-adrenergic receptors has been reported following acute morphine administration (Baddley, Paul, & Carr, 1993) supporting the notion of an intricate relationship between the adrenergic system and opioid-induced immunomodulation. Some immunomodulatory effects of morphine have also been found to be specific to the splenic but not to mesenteric lymph node lymphocytes suggesting a high degree of compartmentalization consistent with SNS innervation of lymphoid tissue (Baddley, et. al., 1993; Lysle, Coussons, Watts, Bennett, & Dykstra, 1993).

Finally, the presence of opioid binding sites on lymphoid cells suggests a direct pathway of immunomodulation. Receptors for opioids are known to exist on lymphocytes (Madden, Donahoe, Zwemer-Collins, Shafer, & Falek, 1987; Carr, DeCosta, Kim,

Jacobson, Guarcello, Rice, & Blalock, 1989; Ovadia, Nitsan, & Abramsky, 1989). These receptors have functional importance in immune homeostasis as indicated by *in vitro* measurement of immunocompetence (for review Carr, 1991).

The focus of this study was to assess the effect of chronic morphine exposure *in vivo* on the effector mechanisms of cell mediated immunity, NK and CTLs measured *in vitro*. A 11 day chronic morphine treatment protocol was developed to investigate this question as well as address the significance of neuroendocrine influences on cell-mediated immunity. The intent of this initial study is to address long term pain management, addiction and withdrawal on immune homeostasis.

MATERIALS & METHODS

Mice and Tumor Lines

Female C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) and C3H/HeN (Harlan-Sprague Dawley, Indianapolis, IN) mice were housed in groups of 6-10 per cage and maintained on a 12 h light/dark cycle. Access to water and food (Purina Mouse Chow) was available *ad lib*. The YAC-1 mouse lymphoma cell line, P815 mastocytoma cell line, and EL-4 lymphoma cell line were obtained from the American Type Culture Collection (Rockville, MD); the cells have been maintained in culture by biweekly passage over the course of no more than 6 months/frozen lot.

Morphine Treatment Regimen

A dose-response study has established that 50.0 mg/kg of morphine subcutaneously (s.c.) results in maximal suppression of NK cytolytic activity (Carr, Gerak, & France, 1994b). In addition, preliminary results from dose effect studies indicates that 50.0 mg/kg elicits maximum suppression of CTL activity in alloimmunized mice (Carr, unpublished observation). This dose was used in all experiments.

In the chronic morphine exposure protocol, C3H/HeN mice (n=23/group) were administered vehicle or morphine 2 h prior to receiving 1 X 10^7 C57BL/6J spleen cells, intraperitoneally (i.p.). Following the immunization, mice received vehicle or

morphine daily for an additional 6 days. On day 7, mice were re-immunized with 1 X 10^7 C57BL/6J spleen cells, i.p. 2 h after the administration of vehicle or morphine. Following the second immunization, mice received morphine or vehicle daily for an additional 3 days. On day 11, the mice were sacrificed and splenic lymphocytes (SL) were recovered and assayed for CTL and NK activity or processed further for other assays described below.

Lymphocyte Preparation

All mice were sacrificed by CO_2 asphyxiation and peritoneal lavage was performed using 10 ml of sterile Hank's buffer saline solution (HBSS). Cells were collected by recovering 10 ml of peritoneal fluid through a 20 gauge needle and 10 ml syringe. Spleens were removed and cell suspensions were prepared by mechanical dispersion. SL and peritoneal exudate leukocytes (PEL) were washed with HBSS (250 x g, 5 min). Red blood cells were osmotically lysed using 0.84% NH_4CL; the cells were subsequently washed with HBSS (250 x g, 5 min) and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS) and 2.5% Hybri-max (Sigma Chemical Co., St. Louis, MO) antibiotic (100 units/ml penicillin; 200 μg/ml streptomycin)/antimycotic (250 ng/ml amphotericin B) solution (complete medium). Cells were counted and examined for viability via trypan blue exclusion dye.

^{51}Cr-Release Cytolytic Assay

CTL activity was assayed using a 4 h microcytotoxicity assay with ^{51}Cr-labeled EL-4 (H-2^b) cells as targets. Between 5 X 10^4 and 160 X 10^4 effector cells were mixed with 1 X 10^4 target cells in conical 96-well microtiter plates (Costar, Cambridge, MA) in a reaction volume of 0.2 ml of complete medium. The cultures were incubated 4 h at 37°C in a 5% CO_2 atmosphere. A 100 μl aliquot of cell-free supernate was taken from each well and its ^{51}Cr content was determined using a Beckman gamma counter. The cytolytic activity was determined as follows: percent cytolytic activity=[(experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(total cell-associated ^{51}Cr release - spontaneous ^{51}Cr release)] X 100 where "spontaneous" refers to ^{51}Cr release by target cells in the absence of effector cells. Total cell-associated ^{51}Cr was determined by measuring the ^{51}Cr content in the supernates of 10^4 target cells incubated at 37°C in a 5% CO_2 atmosphere in the presence of 0.1% t-octylphenoxypolyethoxyethanol (Triton X-100, Sigma Chemical Co.) in complete medium or measuring the ^{51}Cr content in 10^4 ^{51}Cr-labeled target cells. Spontaneous release was consistently between 10-15%. Each effector to target cell ratio was measured in triplicate/animal. One lytic unit (LU) was defined as the number of effector cells required to lyse 20% of the target cells per 10^7 total SL or PEL population. To determine antigen specificity for the CTL assay, P815 (H-2^d) mastocytoma cells were

^{51}Cr-labeled and used as third party targets in the 4 h microcytotoxicity assay.

CTL Serine Esterase Assay

One hundred μl of SL at a concentration of 2 X 10^6 cells/ml from C3H/HeN mice were placed in duplicate 96-well conical bottom microtiter plates. One X 10^5 irradiated (900 rads) C57BL/6J spleen cells in 100 μl of complete media were added to one set of wells. One hundred μl 0.2% Triton X-100 in complete media was added to the other set as a measure of total activity. Cells were incubated for 2 h or 4 h and 10 μl of supernate was removed from each well and transferred to the wells of a separate 96-well flat bottom plate (Costar). To each well 190 μl of substrate consisting of 0.2 mM N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (Sigma Chemical Co., St. Louis, MO) was added. Duplicate wells of complete media and 0.2% Triton X-100 were included as reagent blanks. The plates were incubated for 1 h and the optical density at 405 nm determined in a Dynatech MR5000 automatic plate reader. The esterase activity was determined as follows: percent esterase activity = [(experimental absorbance - complete media blank absorbance)/(total absorbance-0.2% Triton X-100 absorbance)] x 100. Each well was read in triplicate.

$CD8^+$ Lymphocyte Enrichment

Mouse T cell subset enrichment columns (R & D Systems, Minneapolis, MN) were prepared as suggested by the manufacturer.

SL from the groups were pooled as were SL from the morphine treatment groups and separately applied to CD8 enrichment columns. Recovered T cells were then assayed for CTL activity using ^{51}Cr-labeled EL-4 cells as targets in the ^{51}Cr-release cytolytic assay. Unfractionated splenic lymphocytes consisted of 12 ± 3 % $CD8^+$ lymphocytes whereas the $CD8^+$-enriched SL population consisted of 64 ± 5 % $CD8^+$ lymphocytes, 0.0 % $CD4^+$ lymphocytes, and 32 ± 6 % B lymphocytes, macrophages, and null cells as determined by flow cytometry using a Coulter Elite (data not shown).

Serum Corticosterone and Dehydroepiandrosterone (DHEA) Assay

Animals were sacrificed by CO_2 asphyxiation and the thoracic cavity immediately opened and blood extracted via heart puncture with a 1ml syringe and 27 1/2 gauge needle. Samples were placed on ice overnight and exposed to the air to favor clot formation. Tubes were then spun at 10,000 x g for 1 min. in a microcentrifuge (IEC; Needham, MA.). Sera were removed and frozen at -20°C for later assay.

Sera from sacrificed animals were assayed for corticosterone by RIA using a corticosterone kit specific for rat and mouse corticosterone (ICN Biomedicals; Costa Mesa, CA) or DHEA (Diagnostic Products Corp., Los Angeles, CA) All samples were assayed simultaneously in duplicate. Standards were run simultaneously with experimental samples. The concentration of

serum corticosterone and DHEA in the experimental samples was extrapolated from the curve generated from the known standards. The standard curve typically had a corresponding coefficient for linearity >.9900.

Cellular Cyclic Adenosine Monophosphate (cAMP) Assay

One X 10^6 enriched $CD8^+$ lymphocytes in 200 μl of complete media were placed into two Falcon 2054 (Becton Dickinson; Lincoln Park, NJ.) sterile snap cap tubes. To one of these tubes, 200 μl of complete medium was added (effector only, unstimulated); to the other tube was added 10^4 irradiated (900 rads) EL-4 target cells (effector + target, stimulated) in 200 μl of complete medium. A further tube containing 10^4 irradiated target cells in 400 μl of complete medium (target associated cAMP) was also prepared. All tubes were incubated at 37°C/ 5% CO_2 for thirty minutes. Parallel experiments were carried out representing vehicle and chronic morphine treatment groups. Following incubation, cells were immediately assayed for cAMP by enzyme-linked immunoabsorbent assay (ELISA) (ELISA technologies; Lexington, KY.). Standards were run simultaneously with experimental samples. The concentration of cAMP in the experimental samples was extrapolated from the curve generated from the known standards. The standard curve typically had a correlation coefficient for linearity >.9900.

Conjugate Formation Fluorescence Activated Cell Sorter (FACS) Analysis

The protocol for labeling and measuring conjugates was carried out as described (Lebow & Bonavida, 1990) with modifications. Specifically, 5 X 10^5 $CD8^+$ lymphocytes from each treatment group (vehicle and chronic morphine) were placed into Falcon 2054 (Becton Dickinson; Lincoln Park, NJ.) sterile snap cap tubes. An additional tube of 5 x 10^5 enriched cells from vehicle-treated animals was also prepared (effector only). Cells were washed once with 1 ml PBS at 250 g for 5 min, room temperature. Fluorescein isothiocyanate (FITC) dye was prepared by dissolving powdered FITC (Sigma; St. Louis, MO.) in dimethyl sulfoxide (DMSO) at a concentration of 50 mM and diluting 1:1000 in phosphate buffered saline (PBS, pH 7.4). Dye solution (100 μl) was added to the decanted washed cells and the cells then vortexed momentarily before being incubated at 37°C/ 5% CO_2 for 10 mins with the caps dislodged. Cells were then washed twice as before with PBS and decanted. A volume of 250 μl of target cells (EL-4) at a concentration of 4 x 10^6 cells per ml complete media was added to the cells from the experimental groups establishing a effector to target ratio of 1:2 favoring formation of single effector-target conjugates. Complete medium (250 μl) alone was placed into the tube containing effector cells alone and an additional tube containing 250 μl of the target cell suspension was prepared (target alone). Tubes were incubated for 90 mins. at 37°C/ 5% CO_2 with the caps dislodged and occasionally

agitated gently to maintain suspension. Following the incubation, the cells were immediately placed on ice for FACS analysis. Immediately prior to FACS analysis, 5 μl of 0.1 μg/μl solution in phosphate buffered saline (PBS, pH 7.4) of propidium iodide (Sigma; St. Louis, MO.) was added and the sample gently agitated to distribute the dye and insure a uniform cell suspension.

FACS analysis was carried out on a Coulter Elite FACS (Coulter, Hialeah, FL). A log forward scatter vs log side scatter plot was used to gate viable cells for analysis, thereby separating whole cells from cell fragments. Whole effector only cells were plotted log green (FITC) vs. log yellow (propidium iodide) which allowed effectors to be sorted for viability, needed for calculation of % conjugation in subsequent analysis of experimental samples. A log green vs log side scatter plot of effector only was gated so that viable effector cells were counted. The target only sample was then analyzed with the above gates to insure that target alone was not counted in the effector population. The vehicle experimental sample was analyzed and another gate was created to encompass the bulk of conjugates. An analysis window, cell count vs log yellow, was gated to the conjugates to allow for the calculation of % killing within the conjugated population. Analysis of the chronic morphine-treated sample proceeded with the above gate settings.

Calculation of percent conjugation: % conjugation= (# of conjugates / [# viable effectors + # of conjugates]) x 100. Calculation of percent killing: % killing= (# of gated dead cells within conjugate population / total gated conjugates) x 100. Effector cells only yielded 5 ± 2 % uptake of PI. This number was used to subtract out percent killing in the conjugate gate. Target cells only showed less than 1 % PI uptake and were therefore not included in the background substraction.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA Extraction: SL (1×10^7) RNA was extracted by vortexing in 1 ml of UltraSpec (Biotex, Houston, TX.) RNA extraction solution in 1.5 ml microcentrifuge tubes. Samples were placed on ice for 5 min after which 0.2 ml chloroform was added followed by 15 sec of vortexing and another 5 min incubation on ice. Samples were vortexed and then centrifuged for 15 min at 12,000 x g and 4° C. After centrifugation, 80% of the upper aqueous phase was removed and placed into another 1.5 ml microcentrifuge tube. One volume of ice cold isopropanol was added to the aqueous phase and the mixture was vortexed for 10 sec and placed on ice for 20 min. The samples were then centrifuged for 10 min at 12,000 x g and 4° C. Samples were vacuum decanted and washed twice with ice cold 75% ethanol, centrifuging for 10 min at 7500 x g and 4° C after each wash. Samples were then dried under vacuum and resuspended in 75 μl of sterile water. Optical density readings (Beckman DU-

50 spectrophotometer) at 260 and 280 nm of a 1 μl aliquot were taken to determine the concentration and purity of RNA.

Reverse Transcription: RNA samples were diluted to a concentration of 1 μg/ 3 μl water, 3 μl of each were placed into 0.5 ml microcentrifuge tubes suitable for temperature cycling. A RT master mix was prepared with the following reagents (Perkin Elmer Cetus, Norwalk, CT.) at these corresponding final concentrations: $MgCl_2$-5 mM, 10X PCR Buffer- 1X, dGTP- 1 mM, dCTP- 1 mM, dATP- 1 mM, dTTP- 1 mM, RNase Inhibitor- 1 U/μl, RT- 2.5 U/μl, Random Hexamer Primers- 2.5 μM. A 17 μl aliquot of the master mix was added to each of the 1 μg samples. Tubes were submitted to temperature cycling (MJ Research) consisting of 42° C for 15 min. followed by 99° C for 5 min. and 5° C for 5 min.

Polymerase Chain Reaction: A PCR master mix was prepared with the following reagents (Perkin Elmer Cetus) at these corresponding final concentrations: $MgCl_2$-2 mM, 10X PCR Buffer- 1X, Taq DNA Polymerase- 2.5 U/μl, relevant "Upstream" primer 0.15 μM, relevant "Downstream" primer- 0.15 μM. A 80 μl aliquot of the master mix was added to each of the samples which had been reverse transcribed. Tubes were again submitted to temperature cycling of 95° C for 2 min. followed by 35 cycles of 1 min at 95° C and 1 MIN at 65° C followed by 7 min. at 60° C and soak at 4° C.

Primers:

G3PDH

5′-GTC-ATG-AGC-CCT-TCC-ACG-ATG-C-3′	"Upstream"
5′-GAA-TCT-ACT-GGC-GTC-TTC-ACC-3′	"Downstream

POMC

5′-GAG-ATG-AAC-AGC-CCC-TGA-CTG-AAA-AC-3′	"Upstream"
5′-AAT-GAG-AAG-ACC-CCT-GCA-CCC-TCA-CTG-3′	"Downstream"

Route and dose of morphine and Herpes Simplex Virus-I

Vehicle (s.c.) or morphine (50 mg/kg, s.c.) was administered to C3H/HeN mice. Two hours following the drug administration, the LD_{50} of the McKrae strain of herpes simplex virus (HSV)-I (3×10^5 pfu) in RPMI-1640 was administered in the footpad of the mice in a volume of 50 μl. Subsequent to the virus administration, mice received vehicle (s.c.) or morphine (50 mg/kg, s.c.) daily up to the time of death of the animal or until the end of the observation period (21 days).

Reagents

Morphine sulfate was generously provided by the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). Drug was dissolved in dimethyl sulfoxide (DMSO) and diluted with HBSS to a concentration containing 25% DMSO. A volume of 100 μl of this solution containing the drug at the appropriate concentration was delivered to each mouse. Vehicle consisted of 25% DMSO in HBSS.

Statistics

One-way ANOVA (Randomized Block Design) was used together with Scheffé or Tukey's post hoc multiple comparisons test to determine significance ($p<.05$) between vehicle and drug treated groups. In some experiments, Bonferonni's T-test was used to determine significance ($p<0.5$) between vehicle and drug treated groups. In addition, the non-parametric Wilcoxon signed rank test was used to calculate significant differences between the treated groups of animals. This statistical package used the GBSTAT program (Dynamic Microsystems Inc., Silver Springs, MD).

RESULTS

Chronic Morphine Exposure Suppresses CTL Activity

Mice treated with morphine for 11 days exhibited significantly less SL CTL activity compared to vehicle-treated controls (Fig. 1). PEL CTL activity was also significantly lower in the chronic morphine-treated compared to vehicle-treated mice (Fig. 2). However, both vehicle- and chronic morphine-treated mice showed similar levels of splenic NK activity (Fig. 3). SL from chronic morphine- and vehicle-treated mice were also assayed for lysis of a third party target. Neither population of PEL or SL showed any measurable cytolytic activity against ^{51}Cr-labeled P815 cells (data not shown). In addition, SL from unprimed (non-immunized) mice had no measurable CTL activity to the ^{51}Cr-labeled EL-4 targets (data not shown). Consistent with another study (Carpenter & Carr, submitted) splenic $CD8^+$-enriched effector cells taken from mice treated daily with morphine over 11 days showed significantly less CTL activity compared to vehicle-treated controls (Fig. 4).

Serine Esterase Release is Reduced in Response to Antigen in SL From Chronic Morphine-Treated Animals

Serine esterases are contained within the granules of CTLs and are released upon positive contact with target (Pasternack &

Eisen, 1985; Pasternack, Verret, Liu, & Eisen, 1986; Young, Leong, Liu, Damiano, & Cohn, 1986). To further investigate the reduced cytolytic activity found in the chronic morphine-treated animals, serine esterase release and granulation was measured. Serine esterase release from SL in response to alloantigen stimulation yielded results which paralleled the SL CTL activity. Specifically, after 2 h exposure to irradiated targets (C57Bl/6J splenocytes) the percent of serine esterase content in the supernates was significantly lower in the SL taken from chronic morphine-treated mice compared to serine esterase release from the vehicle-treated controls (Table I). In addition, there was a significant decrease in the total serine esterase content of SL taken from chronic morphine compared to vehicle-treated mice at the two hour timepoint (Table I). By four hours post-incubation, the differences were no longer significant (Table I).

Cyclic AMP Levels of Enriched $CD8^+$ Cells From Chronic Morphine-Treated Animals Revealed a Decreased Response to Antigen

Cyclic AMP has been implicated as the second messenger responsible for the termination of attack between effector and target (Valitutti, Dessing, & Lanzarecchia, 1993). A rise in intracellular cAMP has been associated with detachment from the target cell and a halt in degranulation ("preservation of granulation") (Valitutti et al., 1993). Cyclic AMP has also been implicated as the signal responsible for the initiation of

recycling of CTL for subsequent lytic function (Valitutti et al., 1993).

To further examine mechanisms involved in the suppression seen in the chronic morphine-treated animals, measurements of intracellular cAMP in enriched $CD8^+$ cells were made in response to antigen stimulation *in vitro*. Results indicate that the increase in cAMP of $CD8^+$ cells derived from chronic morphine-treated animals is reduced in response to target cells when compared to the response in the $CD8^+$-enriched effector cells from vehicle-treated animals (Table II).

Ability of $CD8^+$ Cells Derived from Chronic Morphine-treated Mice to Form Conjugates is Not Significantly Impaired

In an attempt to correlate conjugate formation with reduced cytolytic activity, a study of the ability of the cells to form conjugates and kill targets was undertaken. The results revealed no significant differences in conjugate formation in the cells derived from chronic morphine-treated versus vehicle-treated animals following a 90 minute incubation period. Of the gated events (viable effector cells conjugated to targets), 45.1 ± 1.8 % were conjugated to target cells of the enriched $CD8^+$ effector cells from vehicle-treated mice compared to 40.7 ± 5.5 % of the effector cells from the chronic morphine-treated group. Of the cells engaged in conjugation, 3.4 ± 1.1 % of the conjugated targets were dead in the vehicle group compared to 3.1 ± 0.7 % in

the morphine group. Consistent with these results, cell surface expression of the CD11a adhesion molecule necessary for the formation of effector-target conjugates was similar in both $CD8^+$ SL from vehicle- and chronic morphine-treated animals (Table III).

Serum Corticosterone Levels Are Significantly Lower After Chronic Treatment With Morphine

To investigate the role of corticosterone in the suppression of cytolytic activity resulting from chronic morphine exposure, sera were assayed for corticosterone in parallel with cytolytic assays of $CD8^+$-enriched cells. Serum corticosterone levels in vehicle-treated animals were significantly elevated compared to morphine-treated mice (Figure 5A). Likewise, DHEA levels were decreased in the chronic morphine-treated mice compared to the vehicle-treated controls (Figure 5B).

Expression of POMC in SL does not Correlate with Chronic Morphine- or Vehicle-treated Animals

Two of the important products of the POMC gene are adrenocorticotropic hormone (ACTH) and opioid peptides (e.g. α, β and gamma endorphins). An alternative source of ACTH may be lymphocytes (Smith, Meyer, and Blalock, 1982). To investigate these cells as a source of immunomodulation, the expression of

the POMC gene in the SL was undertaken. SL RNA from individual animals within the chronic morphine and vehicle experimental groups were submitted to RT. Products of reverse transcription were then amplified by PCR using primers specific for G3PDH and Exon 3 of POMC. Positive control RNA (from AtT-20 corticotroph cell line) was reverse transcribed and amplified in parallel with experimental samples. No pattern could be discerned comparing the drug- to vehicle-treated groups (Figure 6). Specifically, lymphocytes from 3 out of 13 vehicle animals were positive for exon 3 POMC amplified product while the lymphocytes of 6 out of 14 chronic morphine-treated animals screened were positive. All animals had equivalent levels of G3PDH amplified product (Figure 6).

Chronic Morphine Exposure Reduces the Survival Rate of C3H/HeN Mice Infected with Herpes Simplex Virus Type I (HSV-1)

To further define the biological significance of chronic morphine exposure, C3H/HeN mice were infected with an LD_{50} of the McKrae strain of HSV-1. One out of 12 chronic morphine-treated mice infected with the virus survived the 21-day observation period (Figure 7). Moreover, 9 out of 12 morphine-exposed mice had succumbed to the infection within 7 days of virus administration. In comparison, 3 out of 12 vehicle-treated mice survived the virus infection with 6 out of 12 mice succumbing to infection 7 days following virus administration.

DISCUSSION

In the present study, we have investigated the immunomodulatory effect of chronic morphine exposure on NK and CTL activity. Short term (daily exposure to morphine for 5 days) morphine (50.0 mg/kg, s.c.) administration did not modify CTL activity in alloimmunized C3H/HeN mice (Carr, unpublished observation). In addition, the generation of CTLs in one-way mixed lymphocyte reactions is unaffected in the presence of morphine (10^{-5} - 10^{-11} M) (Carr & Carpenter, submitted) suggesting morphine does not directly act on immune cells in the context of CTL generation. However, chronic morphine exposure does suppress CTL activity in alloimmunized mice and this effect is blocked by ß-funaltrexamine (μ-selective opioid receptor antagonist) (Carpenter & Carr, submitted) but not (E)-7-benzylidine-7-dihydronaltrexone (δ-selective opioid receptor antagonist) pretreatment (Carr & Carpenter, submitted). Moreover, the suppression in CTL activity does appear to be modestly significant since only 8% of HSV-I infected mice chronically treated with morphine survived the infection while 25% of vehicle-treated HSV-I-infected mice survived. The reduction in percent survival of vehicle-treated mice following the LD_{50} for this particular strain of HSV-I may be due to the daily handling and injections resulting in a short term "stressed state."

Compartmentalization of morphine induced effects upon indicators of immune function is cited in some studies as evidence for the

involvement of the SNS (Baddley et. al., 1993; Lysle et al., 1993). This appears to be justified given the direct innervation of lymphoid tissue by fibers of the SNS [Felten, Felten, Bellinger, Carlson, Ackerman, Madden, Olschowka, & Livnat, 1987]. The SNS uses norepinephrine almost exclusively as a mediator. The SNS however is not the sole source of norepinephrine. *In vivo*, the adrenal medulla produces epinephrine, dopamine and norepinephrine and is controlled via the SNS as well. In addition, opioids are capable of activating this pathway (Van Loon et al., 1981; Appel et al., 1986). Accordingly, the global immunomodulatory effects of morphine could be mediated by SNS stimulation of the adrenal medulla resulting in the release of sufficient quantities of catecholamines to exert a systemic immunomodulatory effect.

In vitro catecholamines have been shown to have wide immunomodulatory effects on indicators of CTL function. The lytic activity of CTL was found to be potentiated by the addition of NE, EPI or isoproterenol (ß-agonist) at the beginning of culture, co-addition of the ß-blocker timolol abolished the augmentation (Felten et. al., 1987, Livnat, Madden, Felten, & Felten, 1987]. *In vivo*, chemical sympathectomy has been shown to reduce CTL activity and influence lymphocyte trafficking (Madden & Livnat, 1991). Combined the action of catecholamines suggest that their effect is dependent on concentration, timing, cell type and site of action. For example, early effects on CTL

activation and differentiation appear to potentiate activity, whereas late effects inhibit CTL effector function (Strom & Carpenter, 1980).

The effect of endogenous opioids on CTL generation *in vivo* has not been revealed. Unlike morphine, endogenous opioid peptides have been found to potentiate the generation of CTLs *in vitro* through a naloxone-sensitive mechanism (Carr & Klimpel, 1986). To investigate a possible role for these peptides (i.e., endorphins) *in vivo*, we examined the expression of the POMC gene by SL harvested from chronic morphine- and vehicle-treated animals. Results of RT-PCR amplifications of SL RNA failed to reveal a correlation between the expression of exon 3 POMC transcripts and the treatment group. This suggests that the POMC gene may be expressed only transiently in SL and is not an important component in the suppression of cytolytic activity found in the effector population.

The result showing a reduced corticosterone level in the chronic morphine-treated animals relative to vehicle-treated mice is unexpected. Previous studies have shown HPA axis involvement in modifying the immune system following short term exposure to morphine (Bryant et. al., 1991; Sei et. al., 1991; Fuchs & Pruett, 1993). However, the present results would indicate the levels of corticosterone do not coincide with suppression of CTL activity. Moreover, recent results have shown short term

exposure to morphine (50 mg/kg daily for 5 days) *in vivo* has no effect on the generation of CTLs in alloimmunized C3H/HeN mice (Carr, unpublished observation) suggesting the influence of corticosterone in CTL generation or activity is minimal in this strain of mouse. However, in CBAxC57BL/6 mice, short term exposure to morphine (50 mg/kg daily for 5 days) *in vivo* modifies CTL activity (Garza, Prakash, & Carr, submitted) indicating the strain-specific nature of morphine-mediated immunomodulation as previously reported (Bussiere, et. al., 1992) as well as the potential role of the HPA axis.

In the present study, circulating levels of the adrenal cortical steroid hormone DHEA were found to be modestly but significantly lower in the morphine-treated mice. DHEA has previously been shown to protect mice against a lethal dose of virus (coxsackievirus and herpes simplex virus type II) (Loria, Inge, Cook, Szakal, & Regelson, 1988) and is predicted to interfere with the immunosuppressive effects of corticosterone (Riley, 1983). Recently, androstenediol, a metabolic product of DHEA has been found to be 100x more potent than DHEA in regulating resistance to viral and bacterial infections (Loria & Padgett, 1992). In terms of the present study, the results suggest that the increase in HSV-I-elicited encephalitis and death in chronic morphine-treated mice may be due in part to a reduction in the circulating levels of DHEA and metabolites thus eliminating the endogenous corticosterone antagonist. However, the observation

showing corticosterone levels were also significantly lower in the chronic morphine-treated mice seems to suggest the adrenal glands may not been functioning correctly.

Early events in CTL target cell recognition include target cell adhesion followed by "programming" for lysis. Within 2-10 minutes following target cell adhesion cytoplasmic granules within the CTL reorients to the region near the interface with the target cell (Englehard, Gnarra, Sullivan, Mandell, & Gray, 1988). This programming for lysis results from the activation of pathways involving phospholipase C (PLC), phosphatydylinositol turnover and protein kinase C. A sharp increase in intracellular calcium from intracellular and extracellular sources promotes reorientation, fusion and exocytosis of granules (Englehard et al., 1988; Ostergaard & Clark, 1987). Granule fusion is terminated simultaneously with a sharp increase in intracellular cAMP (Valitutti et. al., 1993).

In an effort to identify the mechanism(s) for suppressed cytolytic activity, we investigated the ability of purified CTLs from chronic morphine- and vehicle-treated mice to form conjugates and subsequently lyse targets. A previous study showed acute morphine administration suppressed conjugate formation and cytolysis of target cells by NK-enriched effector cells (Carr et. al., 1994a). The results in the present investigation indicate that the ability of CTLs from chronic

morphine-treated animals to form conjugates with allogenic target cells at the 90 min. timepoint is not impaired. This is consistent with the finding that there were no significant differences in CD11a expression by purified effectors cells between the vehicle- and chronic morphine-treated groups. Differences in CD11a expression would presumably result in differences in the avidity between effector-target conjugates resulting from the nonspecific interactions between CD11a on effector cells and CD54 (ICAM-1) on the target cells (Dustin, & Springer, 1989; Nakamura, Takahashi, Fukazawa, Koyanagi, Yokoyama, Kato, Yagita, & Okumura, 1990; Spits, Schooten, Keizer, Seventer, Rijn, Terhorst, & Vries, 1986). Subsequent killing of targets was likewise not impaired. This result seems contrary to the deficient killing observed in the cytolytic assays. However, the original cytolytic assays were carried out over four hours presumably allowing for multiple effector-target interactions with effectors recycling after initial attack to kill again (Valitutti et. al., 1993). The conjugate studies on the other hand span 1.5 hours, which perhaps is insufficient time *in vitro* for attack and recycling. Measurements made at 2.5-3 hours were inconclusive due to the high background associated with propidium iodide uptake by effector and target cells.

The two groups of enriched CTL, chronic morphine- vs vehicle-treated are not equivalent. One major difference is the granulation found in SL from the chronic morphine-treated

animals. These results suggest that production of esterase-containing granules by the CTL subpopulation is deficient. Assuming that at the time of harvest enriched $CD8^+$ CTL from the chronic morphine- and vehicle-treated animals have both cleared the antigen stimulus, it is probable that the CTL population has recycled and awaits new targets. If at the time of harvest these CTLs represent a population "awaiting" new target then it is logical that the level of CTL granulation present represents a maximum constitutive level in the chronic morphine- and vehicle-treated groups. The 1.5 hour conjugate studies of purified CTL show equivalent ability to form conjugates combined with equivalent capacity to deliver a lethal hit, but killing is impaired in the chronic morphine-treated group in the 4 hour cytolytic assay results. The conjugate results however only represent a primary contact with target, which suggests that the level of granulation present in the "awaiting" CTL from chronic morphine- and vehicle-treated animals are both sufficient to deliver an initial lethal hit although their respective initial level of granulation is different. The subsequent secondary and tertiary contact with the target cells is likely to be where the defect resides. Accordingly, this points to a possible defect in CTL recycling in the chronic morphine-treated animals. The results of the cAMP studies suggest a mechanism for this result. An increase in intracellular cAMP is associated with termination of attack after a positive interaction with target and believed to be the secondary signal that induces detachment from target,

initiation of recycling and preservation of granulation (Valitutti et. al., 1993). The cAMP levels in CTLs from chronic morphine-treated animals after 30 mins. of antigen exposure appears reduced although basal levels in unstimulated cells are similar to that of the vehicle-treated animals. This suggests that the CTL derived from the chronic morphine-treated animals are defective in the termination of attack, and this may prolong CTL contact with target and lead to excessive degranulation and delayed recycling. Together these processes might impair subsequent killing or simply reduce the total number of lytic contacts in the 4 hour time period of the cytolytic assays. In summary, two pathways of chronic morphine treatment are proposed; 1.) Esterase content of CTLs is reduced. 2.) Termination of attack is impaired leading to impaired recycling and excessive degranulation and/or prolonged contact leading to a reduction in total contacts within the time frame of the assays.

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Abbreviations used in this paper:

ACTH, adrenocorticotropic hormone; **cAMP**, cyclic adenosine monophosphate; **CNS**, central nervous system; **CTL**, cytotoxic T lymphocyte; **DHEA**, dehydroepiandrosterone; **DMSO**, dimethyl sulfoxide; **ELISA**, enzyme-linked immunoabsorbent assay; **EPI**, epinephrine; **FACS**, Fluorescence activated cell sorter; **FCS**, fetal calf serum; **FITC**, fluorescein isothiocyanate; **HBSS**, Hank's buffered saline solution; **HSV-I**; herpes simplex virus type-I; **HPA**, hypothalamic-pituitary adrenal; **ICAM** intra-cellular adhesion molecule; **IFN**, interferon; **IL**, interleukin; **i.p.** intraperitoneal; **LU**, lytic unit; **NE**, norepinephrine; **NK** natural killer; PEL , peritoneal exudate leukocytes; **PBS**, phosphate buffered saline; **PCR**, polymerase chain reaction; **PLC** phospholipase C; **POMC**, Proopiomelanocortin; **RT**, reverse transcription; **s. c.**, subcutaneous; **SL**, splenic lymphocyte; **SNS**, sympathetic nervous system; **Triton X-100**, t-octylphenoxypolyethoxyethanol.

Footnotes

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Figure Legends

Figure 1. Chronic morphine exposure suppresses splenic lymphocyte CTL activity. C3H/HeN mice (n=23/group) were administered morphine (50.0 mg/kg, s.c.) or vehicle 2 h prior to alloimmunization (1 X 10^7 C57BL/6 SL, i.p.). Mice received morphine (50.0 mg/kg, s.c.) or vehicle daily for 9 days and were re-immunized 7 days after the primary immunization. Mice were sacrificed on day 11 and the SL were assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. $^{*}F(1,22)=15.455$, $p<.05$ comparing vehicle- to chronic morphine-treated mice as determined by one-way ANOVA and Scheffe multiple comparison test. Bars represent SEM, n=23.

Figure 2. Chronic morphine exposure suppresses CTL activity of peritoneal exudate leukocytes. Mice were treated as described in the legend of Fig. 1. PEL were collected and assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. $^{*}F(1,22)=6.3882$, $p<.05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA followed by Scheffe multiple comparison test. Bars represent SEM, n=23.

Figure 3. Chronic morphine exposure has no effect on splenic NK activity. Mice were treated as described in the legend of Fig. 1. SL were assayed for NK activity using ^{51}Cr-labeled YAC-1 cells. Bars represent SEM, n=23.

Figure 4. Chronic morphine exposure suppresses $CD8^+$-enriched CTL activity. C3H/HeN mice (n=4/group) were treated as described in the legend of Fig. 1. Mice were sacrificed on day 11 and the SL wee pooled within treatment groups, enriched for $CD8^+$ cells and assayed for CTL activity against ^{51}Cr-labeled EL-4 cells. $^*F(1,5)=6.5794$, $p<.05$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffe multiple comparison test. Bars represent SEM, n=4.

Figure 5. Chronic morphine exposure reduces serum corticosterone and serum DHEA levels. C3H/HeN mice were treated as described in the legend of Fig. 1. Upon sacrifice, blood was obtained by cardiac puncture and assayed for corticosterone and DHEA levels by radioimmunoassay. A. Analysis of serum corticosterone levels, $^*p<.05$ comparing vehicle- to chronic morphine-treated mice as determined by Bonferroni's T-test. Bars represent SEM, n=11 (vehicle) or 13 (chronic morphine) animals tested. B. Analysis of serum DHEA levels. $^{**}F(1,29)=32.0516$, $p<.01$ comparing vehicle- to chronic morphine-treated mice as determined by ANOVA and Scheffe multiple comparison test. Bars represent SEM, n=15/group.

Figure 6. POMC transcript levels do not coincide with morphine-mediated suppression of splenic CTL activity. SL RNA was subjected to RT-PCR using oligonucleotide probes specific for exon 3 of POMC or G3PDH as described in the Methods section. Lanes 1-4, RNA from SL from vehicle-treated mice; Lane 5, DNA ladder in descending order: 1000 bp, 700 bp, 500 bp, 400 bp, 300 bp, and 200 bp; Lanes 6-8, RNA from SL from chronic morphine-treated mice; Lane 9, RNA from AtT-20 pituitary tumor cells. (A) RT-PCR amplification using POMC primers resulting in a 529 bp product. (B) RT-PCR amplification using G3PDH primers resulting in a 238 bp product.

Figure 7. The effect of chronic morphine treatment on the percent cumulative survival of C3H/HeN mice following HSV-I infection. Mice were injected into the footpad with the LD_{50} of the McKrae strain of HSV-I 2 h after receiving vehicle or morphine as described (see Materials & Methods). $p<.001$ comparing morphine-treated to vehicle-treated mice % survival as determined by the non-parametric Wilcoxon signed rank test, $z=8.29$.

TABLE I. MORPHINE SUPPRESSES THE RELEASE AND TOTAL CELL-ASSOCIATED SERINE ESTERASE (SE) LEVEL IN CTL CELLS[a]

TREATMENT	SE CONTENT IN SUPERNATANT[b]		CELL-ASSOCIATED SE[c]	
	2-h	4-h	2-h	4-h
VEHICLE	13.3±2.2	25.5±4.7	.097±.008	.046±.007
MORPHINE	8.4±1.9*	18.4±3.7	.066±.002*	.039±.004

[a]Splenic lymphocytes from immunized mice were assayed for SE content following re-exposure to antigen.

[b]Cell-free supernatant from mixed lymphocyte reactions were collected and assayed for SE at the designated time points. The numbers represent the % of the total cell-associated SE. Splenic lymphocytes from unprimed C3H/HeN mice were used to determine baseline SE levels. The baseline levels were subtracted from the experimental percentages.

[c]Total cell-associated SE was determined following lysis (0.1% Triton X-100 in complete medium) of the C3H/HeN splenic lymphocytes. Numbers are in absorbance read at 405 nm (background subtracted).

*$F(1,11)=6.3307$, $p<.05$ for the % SE content in supernatant; $F(1,4)=9.9415$, $p<.05$ for cell-associated SE comparing morphine- to vehicle-treated controls as determined by one-way ANOVA and Scheffe multiple comparison test.

TABLE II. CHRONIC MORPHINE EXPOSURE ATTENUATES cAMP PRODUCTION FOLLOWING ANTIGEN STIMULATION IN CD8$^+$ EFFECTOR CELLS

EXPERIMENT	TREATMENT	EFFECTOR ONLY	EFFECTOR + TARGET
#1	VEHICLE	8.9 ± 1.8[a]	13.2 ± 0.8
	MORPHINE	10.3 ± 1.7	10.7 ± 2.1
#2	VEHICLE	9.1 ± 0.1	11.0 ± 0.7
	MORPHINE	5.0 ± 1.9	5.0 ± 2.0
#3	VEHICLE	10.1 ± 0.7	16.9 ± 2.8
	MORPHINE	9.0 ± 0.7	14.1 ± 3.7
#4	VEHICLE	6.2 ± 0.9	11.6 ± 0.5
	MORPHINE	5.6 ± 0.8	6.5 ± 0.6
SUMMARY	VEHICLE	8.6 ± 0.8	13.2 ± 1.3*
	MORPHINE	7.5 ± 1.3	9.1 ± 2.1

[a]Numbers are expressed in pmols/10^6 effector cells ± SEM, n=4. Target cells alone yielded 1.1 ± 0.5 pmols/10^4 cells.

*$F(3,15)=2.9738$, $p<.05$ comparing effector only to effector + target in the summary vehicle group as determined by one-way ANOVA and Tukey's post hoc T-test.

TABLE III. EXPRESSION OF CD11a ON $CD8^+$ LYMPHOCYTES IS NOT ALTERED AFTER CHRONIC MORPHINE EXPOSURE

TREATMENT	PERCENT $CD11a^{+a}$	PERCENT $CD11^+CD8^+$
VEHICLE	75.9 ± 0.6	10.5 ± 0.6
MORPHINE	81.8 ± 0.9*	12.5 ± 0.6

[a]Total percentage of $CD11a^+$ expressing splenic lymphocytes ± SEM, n=6 vehicle-treated, n=5 morphine-treated.

*$p<.05$ comparing morphine- to vehicle-treated mice as determined by Bonferonni's T-test.

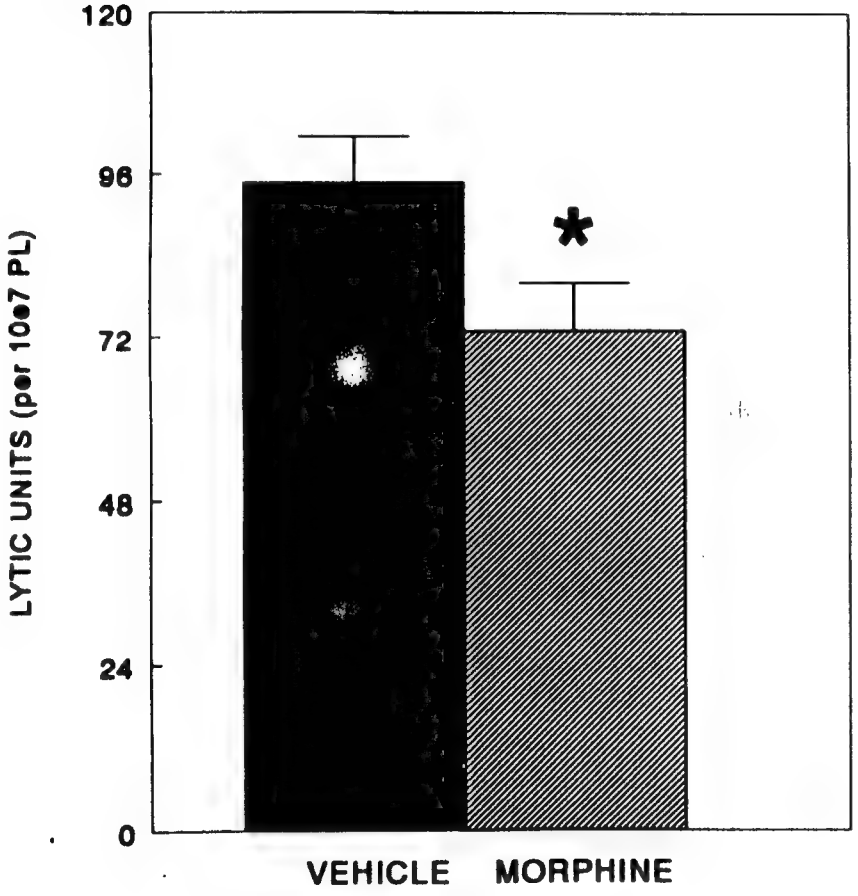

Figure 2

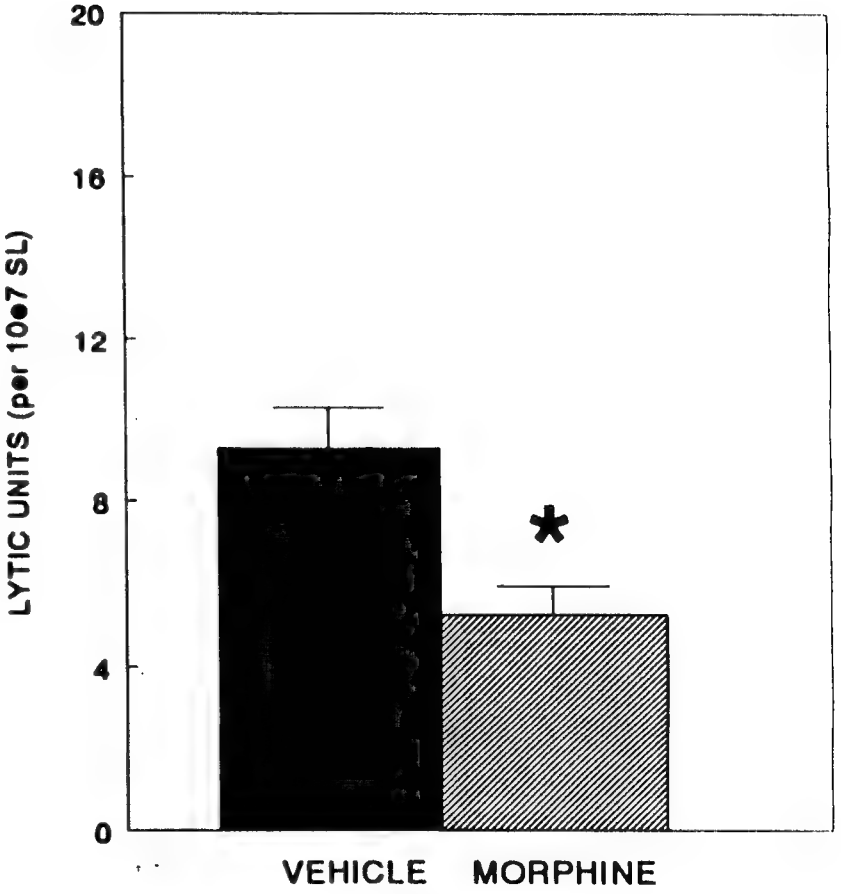

Figure 1

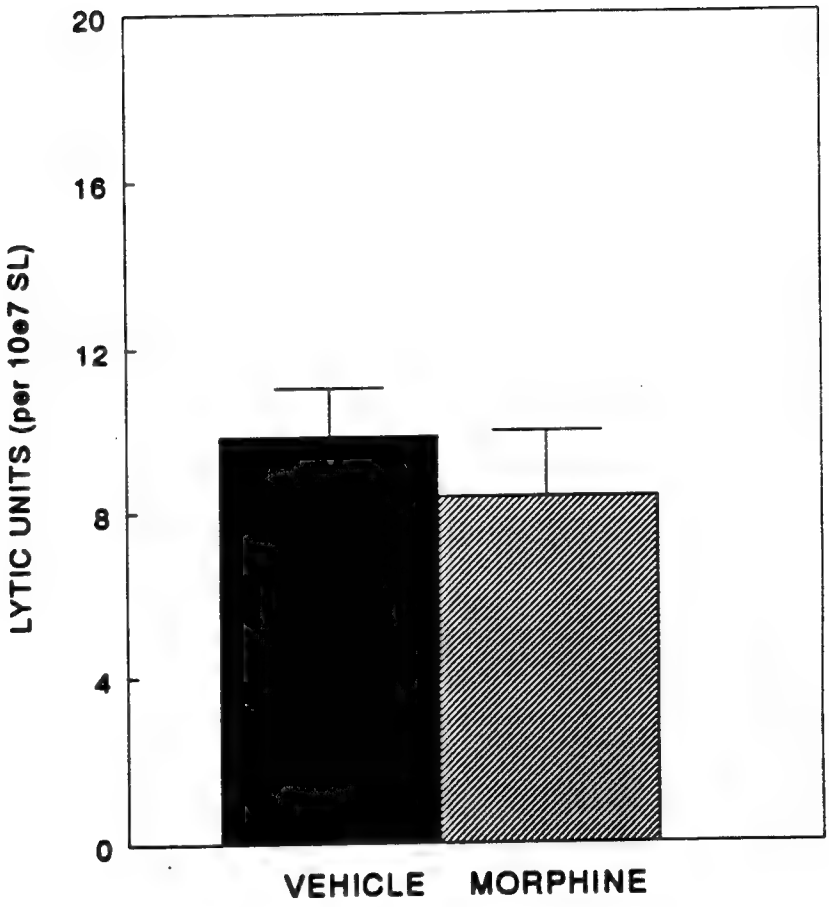

Figure 3

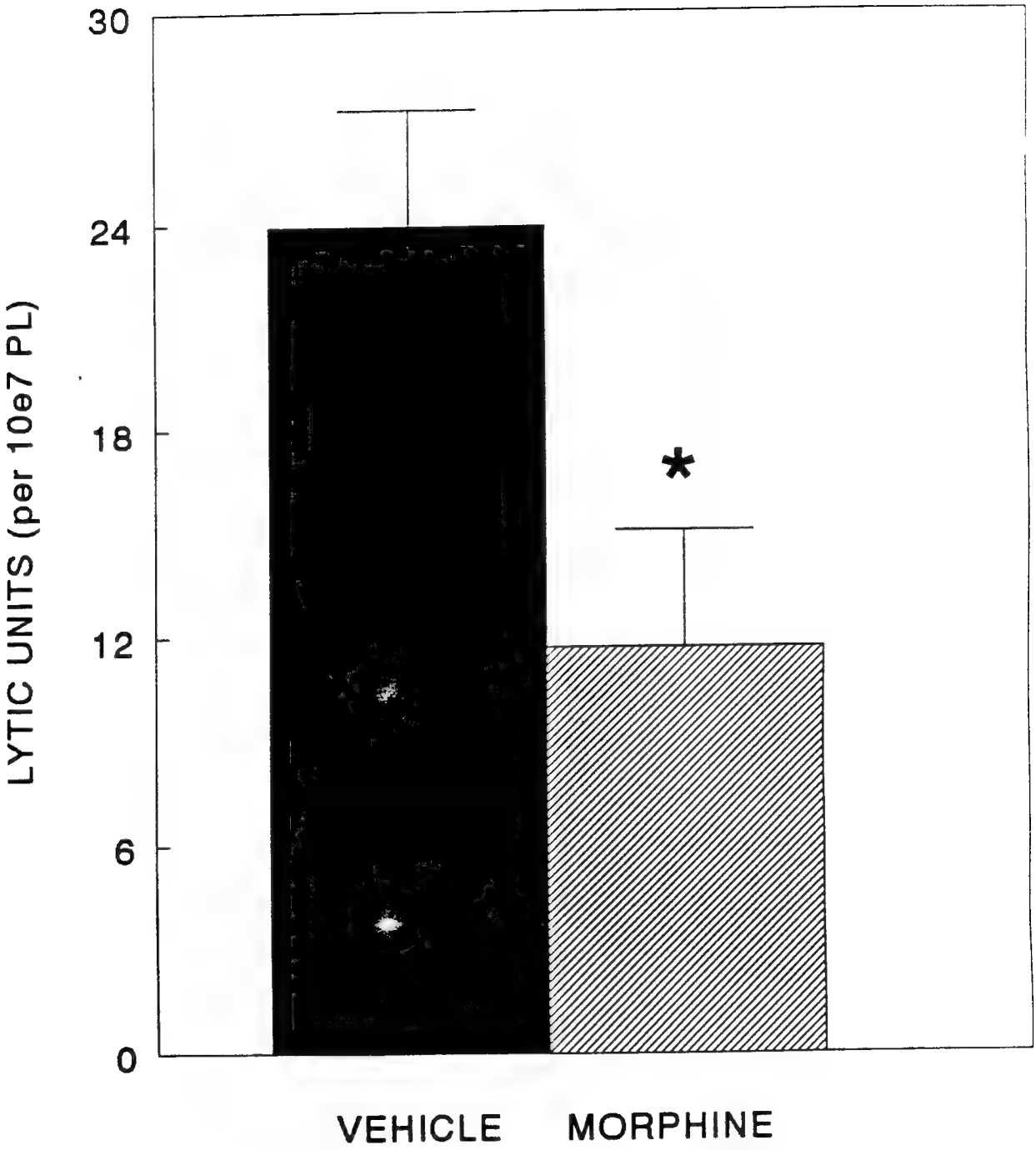

Figure 4

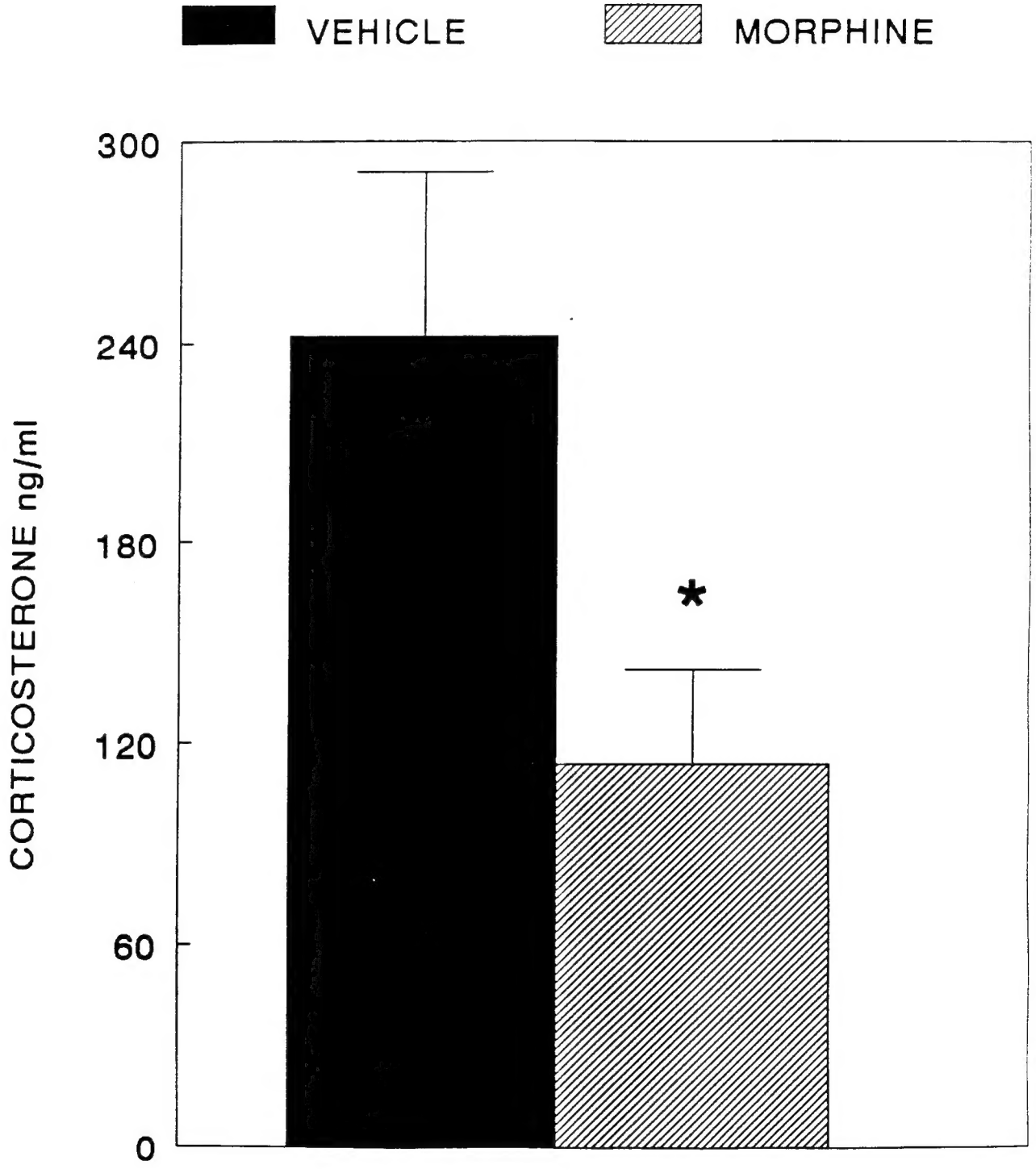

Fig. 5 A

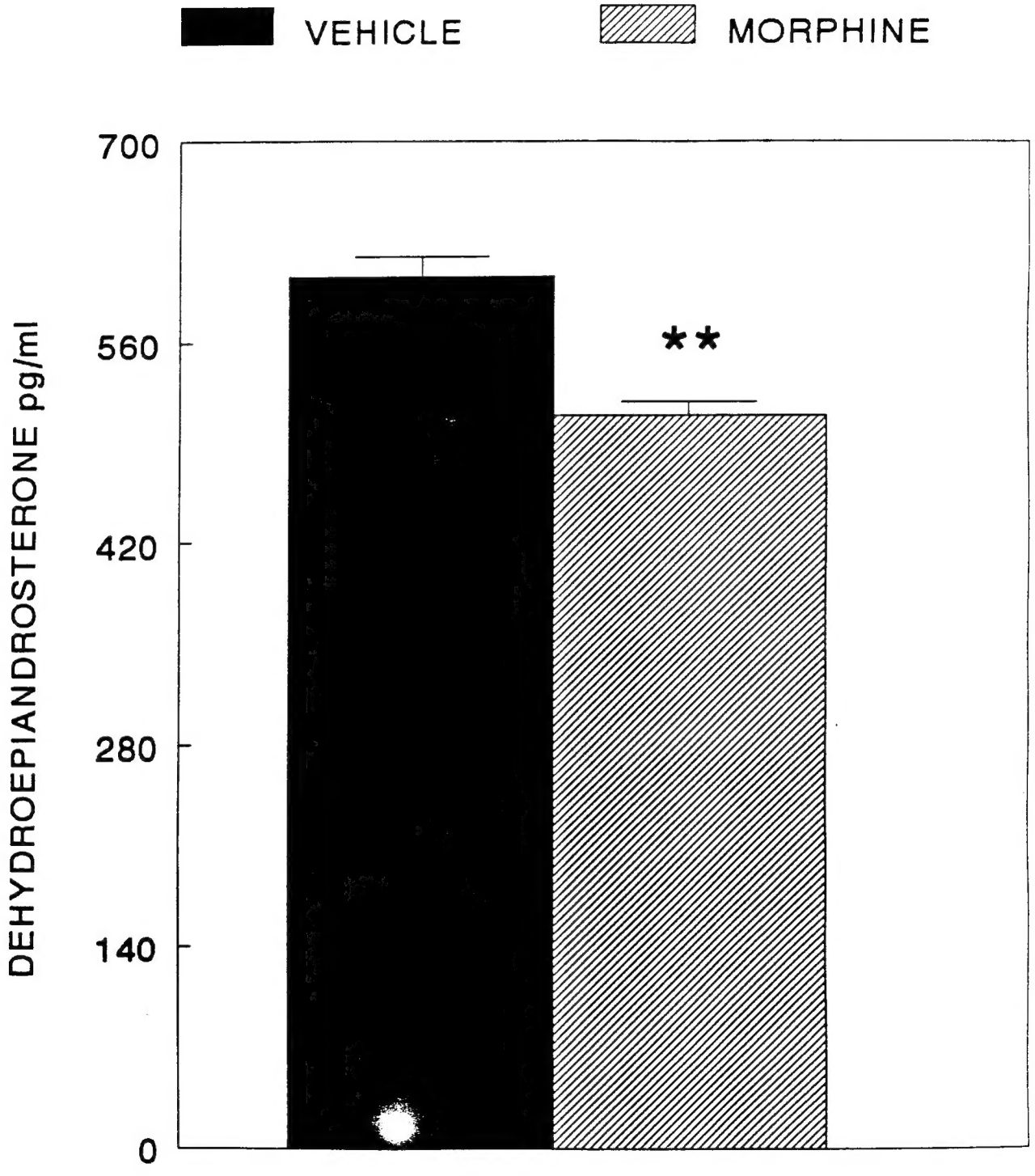
VEHICLE
MORPHINE
700
560
420
280
140
0
DEHYDROEPIANDROSTERONE pg/ml
**

Figure 5B

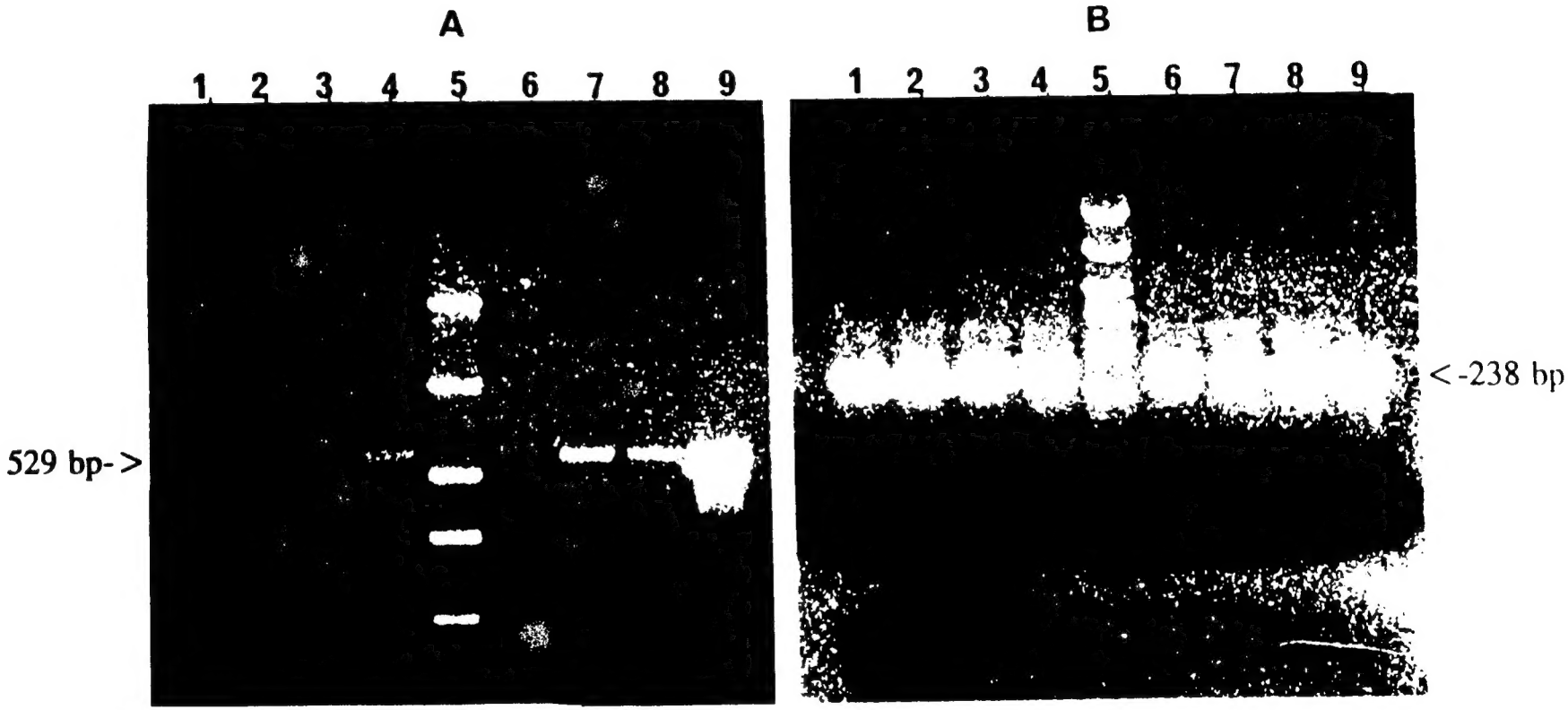

Figure 6

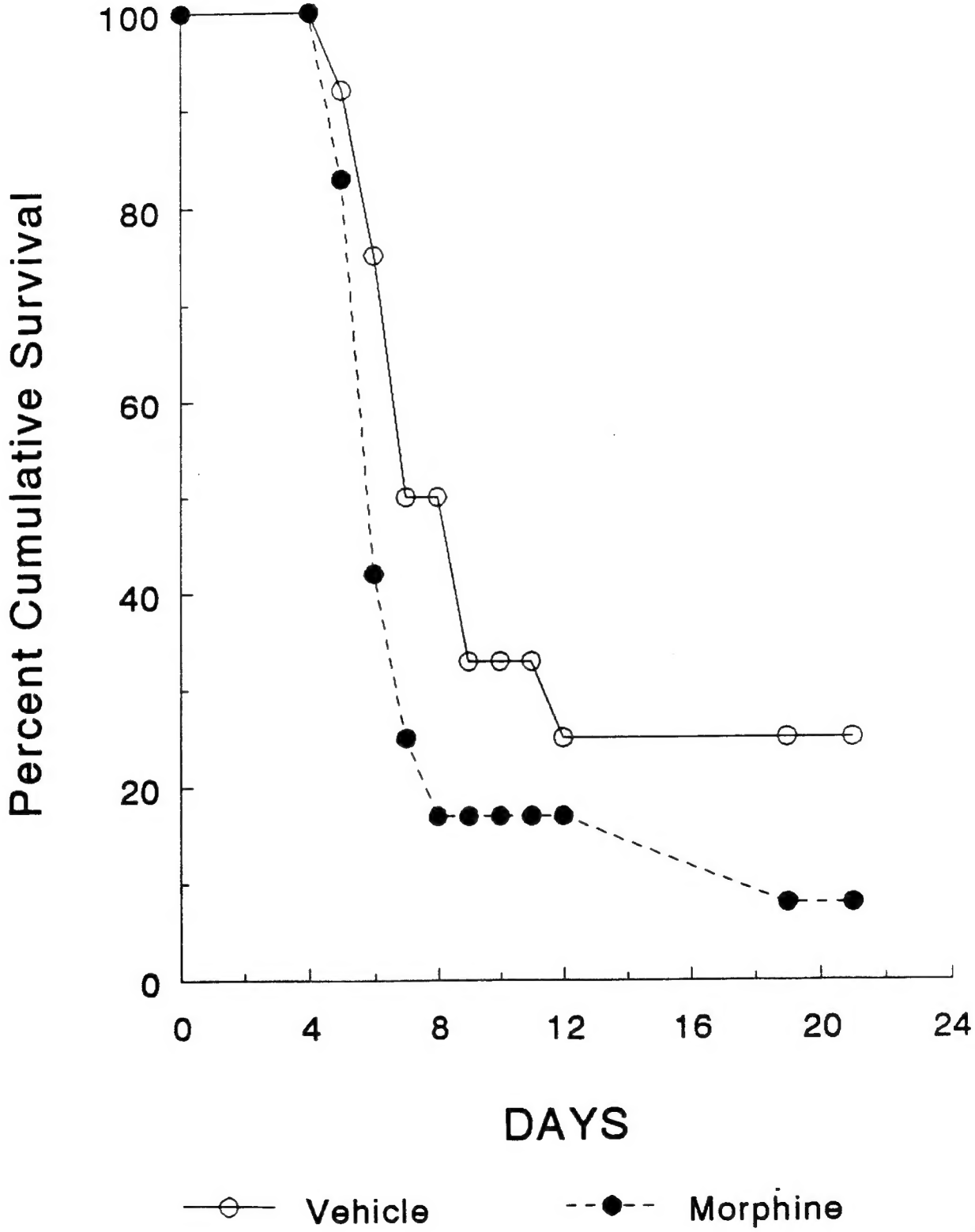
Percent Cumulative Survival
100
80
60
40
20
0
0
4
8
12
16
20
24
DAYS
Vehicle
Morphine

Figure 7